IMMUNOTOXIC EFFECTS OF AMMONIUM METAVANADATE ON MOUSE PERITONEAL MACROPHAGE BIOCHEMISTRY: IMPLICATIONS FOR HOST RESISTANCE TO *LISTERIA MONOCYTOGENES*

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1988

ACKNOWLEDGEMENTS

I wish to express my deepest thanks to my doctoral committee chairman and major advisor, Dr. Cheng-i Wei, to my cochairperson, Dr. Rachel B. Shireman, to my minor advisor, Dr. Lori O. Lim, and to the other members of my committee, Drs. Robert Cousins, Kuo-Jang Kao and Claude McGowan for their support, patience and guidance throughout this project. I am grateful to the Food Science and Human Nutrition Department and the USDA Fellowship committee for providing me with my funding for the years of doctoral research.

I would also like to extend my thanks to all the faculty, staff, and students of the department for all the assistance and cooperation in helping me to complete this dissertation research. Special thanks go to my fellow doctoral candidates in the lab, Joe Owusu-Yaw and Haejung An, and to my co-workers who were immensely helpful in the completion of several of the experiments: Sam Fernando, Dr. Jenny Zhao, Chun-ming Chen, Paul Chang, and Hsiokun-Tan. My greatest and everlasting gratitude goes to Dr. Asok Sen, whose vantage point from physical chemistry allowed interpretations of data that I never would have suspected and who always was willing to discuss possible projects and

the future after graduation. I would be remiss if I did not acknowledge Mr. Walter M. Jones, whose technical assistance in the preparation of seminar slides and many of the figures in this dissertation cannot go unmentioned. All of these people were not only good workmates but good friends over the years, and I am appreciative.

Finally, I am grateful to my parents, brothers and sisters, and their families for their support and encouragement over the years. Their non-science viewpoints and cajoling were often insightful and helped inspire me. I am also indebted to my cohabitants, Sam and Vaska, for providing me humor and companionship without ever having to say a word.

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LIST OF ABBREVIATIONS

BW	Body weight
CMI	Cell-mediated immunity
CM(-)	Complete medium without antibiotic
CMEM	Complete medium
G	Grams
GSH	Reduced glutathione
GSHPX	Glutathione peroxidase
G-6-P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GSSG	Oxidized glutathione
GSSG-R	Glutathione reductase
HBSS	Hanks' balanced salt solution
HMS	Hexose monophosphate shunt
H/T	HBSS:Triethanolamine buffer
IP	Intraperitoneal
LD	Lethal dose
NBT	Nitroblue tetrazolium chloride
NEM	N-Ethylmaleimide
NH_4Cl	Ammonium chloride
NH_4VO_3	Ammonium metavanadate
PAM	Pulmonary macrophage(s)

PBS	Phosphate buffered saline
PEM	Peritoneal macrophage(s)
PMN	Polymorphonuclear leukocyte
SOD	Superoxide dismutase
TE	Triethanolamine buffer
TSA	Trypticase soy agar
TSB	Trypticase soy broth
V	Elemental vanadium

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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August, 1988

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Vanadate-induced immunomodulation had been characterized in an earlier study using the $B_6C_3F_1$ mouse model. Host resistance to challenge with viable *Listeria monocytogenes* and the phagocytic activity of peritoneal macrophages harvested from ammonium metavanadate-treated mice were both decreased in a dose-dependent manner. The present study analyzed the effects of vanadium exposure on enzymes involved in energy production for phagocytosis and in oxygen metabolite formation and detoxification in resident peritoneal macrophages. The effects on intracellular glutathione status were also assayed. Macrophage uptake and intracellular killing of viable *Listeria* as well as the production of superoxide anion were measured following

vanadate pretreatment of mice for six weeks. An *in vitro* analysis of the effects of vanadate ions on those enzymes studied in the intact macrophages was also performed. From the results of the studies, a preliminary mechanism for vanadium immunotoxicity *in vivo* was proposed.

The studies indicated that pretreatment with vanadate resulted in dose-dependent decreases in the ability of the resident macrophages to phagocytize the challenging *Listeria* and decreased intracellular killing activities. Production of superoxide anion necessary for killing *Listeria* was also significantly reduced. The activities of the enzymes glucose-6-phosphate dehydrogenase and glutathione reductase, both essential for phagocytic activity energy production, were decreased by vanadate pretreatment. The activity of glutathione peroxidase was significantly reduced only after host exposure to high levels of vanadate. Intracellular levels of oxidized glutathione were increased although the levels of total glutathione (reduced and oxidized forms) were unaffected. Inhibition of the dehydrogenase was shown to be due to complexation of vanadate with the $NADP^+$ cofactor. Inhibition of the reductase by vanadate was primarily due to a charge-transfer complexation with the oxidized glutathione substrate and most likely with active site disulfides.

Vanadate treatments altered the activities of enzymes needed for energy metabolism and oxygen activation in peritoneal macrophages by affecting the interactions with and cellular levels of necessary substrates and cofactors. These enzymatic dysfunctions hindered phagocytic activity and intracellular killing processes thus allowing continual bacterial replication and, ultimately, host death.

CHAPTER I
INTRODUCTION

The immune system is a complex system composed of several interdependent cell types each of which displays distinct biochemical characteristics. The two major cellular components are the lymphocytes and the macrophages; they are the major participants in the two classes of immune response, cell mediated (CMI) and humoral immunity. Lymphocytes and macrophages develop from pluripotent stem cells originating in the bone marrow. Each cell is released into the circulation in a premature form and migrates to a specific immune system organ (i.e. thymus or spleen) to complete maturation. Once the cells have developed their surface recognition antigens and receptors, they are fully capable of participating in immune responses following proper signalling by biochemical mediators. As lymphocytes proliferate following macrophage-mediated antigenic activation, lymphokines are released which may stimulate macrophage activity to amplify the response. Conversely, suppressive agents may be released to promote an autoregulatory effect such that the immune system is not overused for any one challenge. This interdependence of the major cellular components in the immune system presents a difficult challenge when the possible immunotoxicologic effect of any test agent is studied.

It has been observed that pretreatment of mice with ammonium metavanadate has an immunomodulating effect. Host resistance to bacterial lipopolysaccharide lethality was increased while that to viable Listeria monocytogenes was decreased in a dose-dependent manner. An examination of non-immune indices such as the phagocytic activity/capacity of harvested peritoneal macrophages and the rosetting activity of splenic lymphocytes suggested modification of lymphocyte binding of complement and decreased macrophage functionality without effects on viability. Thymic shrinkage and splenic enlargement were also noted, but histopathologic analysis indicated no direct damage to these organs.

Although there were adequate explanations for each of the observations, the sharp decrease in resistance to Listeria was intriguing. Removal of this pathogen from the body is primarily through CMI mechanisms, although antibodies are also produced during the response. It was hypothesized that the increased lethality of the bacteria was due to (1) a decreased phagocytic activity in macrophages in vivo such that removal of the bacteria is slowed and allows for rapid bacterial proliferation, (2) normal phagocytic activity but decreased digestive/bactericidal processes which allow the survival and ultimate intracellular replication of the Listeria, or (3) some interference in the intercellular messenger system following macrophage ingestion and processing so that lymphocyte responses are decreased.

Therefore, the objectives of this study were (1) to determine the effects of vanadium on the macrophage role in resistance to Listeria, (2) to investigate the manner in which vanadium can disturb macrophage phagocytic and/or digestive functions, and (3) to develop a mechanism by which vanadate can inhibit certain enzymes crucial to energy production and oxygen metabolite detoxification using the peritoneal macrophage as a model system.

CHAPTER II
LITERATURE REVIEW

Vanadium: Chemistry and History of Exposure

Although first discovered by del Rio (who believed he had simply rediscovered chromium), Sefstrom in 1831 became the first person to isolate an oxide of a metal which he termed vanadium in honor of Vanadis, the Norse goddess of love and beauty (Chasteen, 1983). Vanadium is a Group VB transition element, and as such can exist in multiple valence states (0, +2, +3, +4, or +5) in both anionic and cationic forms. The V^{+4} and V^{+5} are the most stable forms and are readily encountered under physiological conditions. Divalent vanadium (V^{+2}) is too strong a reducing agent to exist in any known organism, while V^{+3}, although also a strong reducing agent, is encountered in the specialized vanadocyte cells (with unknown function) in tunicates (Macara et al., 1979a, 1979b; Tullius, 1980). Free elemental vanadium is not known to exist in nature, and the metal is usually found as oxides, sulfides, halide salts or in the form of metallo-complexes (Selbin, 1965).

Vanadium is the tenth most abundant of the elements thought to have biological roles, and the twenty-first among all elements found in the earth's crust (Goldschmidt, 1958; Hopkins et al., 1977). Soil levels range from 3-310 ppm (Bertrand, 1960; Cannon, 1963), with an average of 135-150 ppm (Waters, 1977; Nechay, 1984). The highest levels of

vanadium are found in titaniferous magnetite ores (7000 ppm) and in shales and clay (Faulkner-Hudson, 1964). Resulting crude oils can contain 0.07%-1.0% vanadium by weight (Maylotte et al., 1981), and ashes of these oils contain 65%-85% vanadium (McTurk et al., 1956). Exposure to the dusts during boiler operation or cleaning led to the establishment of occupational threshold limits: 0.5 mg vanadium/m^3 (as vanadium pentoxide) and 1.0 mg/m^3 for the dust and fume forms, respectively. Although workers in the chemical, mining, and steel industries have the greatest risk of exposure to vanadium dusts, high ambient air levels of vanadium (> 0.1 mg V/m^3) often occur in metropolitan areas. Most of the pollutant vanadium, in the form of vanadium pentoxide, ferrovanadium, vanadium carbide or ammonium metavanadate, is the product of the generation of electricity or the burning of coal or oil for heating processes. Thus, ambient air concentrations of vanadium tend to be seasonal, with winter levels three times greater than in the summer months (National Air Sampling Network, 1969).

Symptoms of poisoning in vanadium-exposed workers were first reported by Dutton (1911). The types of illnesses observed in these workers paralleled those seen in patients that received vanadium as a "therapeutic agent" for anemia, chlorosis, syphilis and sciatica (Jackson, 1912) or those who had been wearing clothes dyed with vanadium salts. Dutton noted increased incidences of prolonged coughing spells, tuberculosis, tremors, headaches, as well as a

general irritation of the respiratory tract. Post-mortems on vanadium-exposed workers showed extensive lung and kidney damage, and although vanadium was a factor in the deaths, the primary cause was often respiratory failure secondary to bacterial infections. In the studies of Proescher and Seil (1917), it was claimed that the only true therapeutic uses of vanadium in experimental animals was against trypanosomes and spirochetes (i.e. syphilis); however, the majority of test animals succumbed to secondary infections such as tuberculosis and diplococcus.

Symanski (1939) noted that metallurgical workers exposed to vanadium often exhibited severe pharyngitis, rhinitis, persistent cough, and tightness of the chest. Contrary to Dutton (1911), Symanski claimed that the toxic effects of vanadium were localized and not systemic. Wyers (1946, 1948) demonstrated that a worker with symptoms of bronchitis could recover by removal from the vanadium-containing environment. However, Wyers also noted that many vanadium-exposed workers often developed bacterial infections such as pneumonitis and bronchopneumonia, and Sjoberg (1950, 1955, 1956) reported a significant increase in the incidence of tracheal inflammation, chronic bronchitis, emphysema, and severe chest colds in the workers as compared with a control population. An analysis of respiratory system biopsies showed cytological alterations (Kiviluoto et al., 1979) and a decrease in the immunoglobulin production by circulating plasma cells (Kiviluoto et al., 1981).

Several published reports showed a correlation between chronic exposure to vanadium dusts and an increased susceptibility to colds, influenza, pneumonia, and other respiratory diseases (Roschin, 1967; Schumann-Vogt, 1969; Troppens, 1969). Mice intermittently exposed to metavanadate for short periods (3-6 weeks) have altered resistance to bacterial challenge and to bacterial toxins (Cohen et al., 1986). In a epidemiological study by Stock (1960), a positive correlation between vanadium exposure and mortality from lung cancer, pneumonia, and/or bronchitis was demonstrated. Few studies on the carcinogenic effect of vanadium have been performed, but a life-time study in which mice were fed vanadium at levels below that of the daily human intake (0.116 mg/day) indicated no increase in the numbers of spontaneous tumors (Kanisawa and Schroeder, 1967). However, of the tumors that did form, a greater percentage were malignant and often found in the lungs or as leukemias.

Immunotoxicology

The use of immunological and host resistance assays following exposure to various chemical agents has shown that some agents were toxic to the immune system. In the development of toxicological profiles of chemicals or known toxicants, the concept of chemically-induced alterations of immune function, immunotoxicology, has only recently been emphasized. Following the Michigan polybrominated biphenyl (PBB) incident of 1973, Bekesi et al. (1978) reported

disruptions in the lymphocyte function of local dairy farmers. This report appeared soon after a major comprehensive review by Vos (1977) which outlined much of the reported information linking chemical exposure with altered immune function. With an enhanced interest in studying these effects, immunologists coined the term "immunotoxicology" in order to differentiate themselves from immunopharmacologists who studied chemical agents for their therapeutic potential (Dean et al., 1982a). As a result, the field of immunotoxicology was developed under the basic premise of determining whether the immune system serves as a target organ for injury by a particular chemical under analysis. The immune system may be directly affected or altered indirectly by chemically-induced stress (Norbury, 1985), both of which can potentially be adverse to an exposed host (Figure 1).

To determine the immunotoxicological profile of a test agent, several aspects of immune function must be examined. The classical division of immunity into the humoral and cell-mediated immune (CMI) systems provides the starting point for such studies. Current procedures outlined by the National Institute of Environmental Health Sciences and the National Toxicology Program for defining immune alterations encompass studies of pathotoxicology (hematology profile, clinical chemistries, organ weight and histology), humoral immunity profiles (immunoglobulin levels), bone marrow colony-forming capacity, macrophage function, lymphocyte

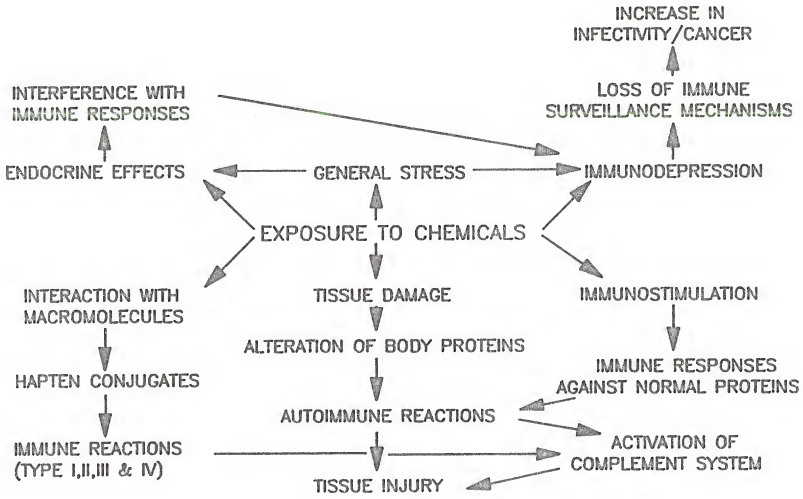


Figure 1. Potential effects of chemicals on the immune system.

proliferation, and cell-mediated function (delayed-type hypersensitivity, graft vs host reactions, tumoricidal assays) (Dean et al., 1982b). The rationale for such a wide variety of recommended studies is the complexity of the immune system. No one assay can adequately describe the overall effect of a test agent. By obtaining a more complete profile, researchers may develop mechanisms of immunotoxicity.

Many classes of chemical compounds have been analyzed for their immunomodifying effects in vivo and in vitro. Most commonly they are agents which pose a risk to human health as pollutants and display a persistence in both the environment and in biological tissues. The classes well studied include the halogenated aromatic hydrocarbons (Nicholson and Moore, 1979; Kimbrough, 1980) such as polychlorinated and -brominated biphenyls (PCB; Thomas and Hindsill, 1978; PBB; Bekesi et al., 1978), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; Vos and Moore, 1974; Luster et al., 1980); the polycyclic aromatic hydrocarbons (PAH; Ball, 1970) such as 3-methylcholanthrene (Malmgren et al., 1952; Stjernsward, 1965), 7,12-dimethyl-benzathracene and benzo(a)pyrene (DMBA and B(a)P); Stjernsward, 1966); and phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA; Grim et al., 1980; Dean et al., 1982a).

Pesticides as a class of immunotoxicants have been well studied (Ercegovich, 1973) with particular interest focused on DDT (Wassermann et al., 1969) and many organophosphorous

agents such as parathion (Street and Sharma, 1975), dichlorvos (Desi et al., 1980), lindane and malathion (Desi et al., 1978). Agents used for protecting livestock health and enhancing growth such as orthophenylphenol (OPP; La Via and La Via, 1979) and diethylstilbestrol (DES; Dean et al., 1980) have also been analyzed due to the increased risk of human consumption. Other agents to which man may be exposed are chemotherapeutic agents such as methotrexate, cyclophosphamide and 6-mercaptopurine. All of these have been shown to be immunosuppressive due to the non-proliferative nature of their action upon tumor and other dividing cells (Hersch, 1974; Bach, 1975; Zschiesche, 1975).

Many metals have been studied for their effects on immune system function and several extensive reviews have been published (Treagen, 1975; Sunderman, 1977; Koller, 1980). Because of previously observed toxic effects in man following inadvertent exposure, lead, cadmium and mercury have undergone the most intense immuntoxicologic analysis.

Lead has been shown by Selye and colleagues (1966) to enhance by 1000-fold the host susceptibility to bacterial endotoxin lethality. The decreased resistance was the result of lead-induced impairment of hepatic and splenic macrophage phagocytosis and antigenic processing (Trejo et al., 1972; Filkins and Buchanan, 1973). These defects in leukocytic activity resulted in the decreased resistance of lead-treated rodents to challenge with Salmonella typhimurium (Hemphill et al., 1971), Escherichia coli (Cook

et al., 1975), and Candida (Salari et al., 1975). Activation of a challenging viral pathogen (i.e. Rauscher leukemia, psuedorabies, encephalomyocarditis) with a simultaneous decrease in host resistance following lead exposure was ascribed to interference in the synthesis of interferon (Gainer 1973, 1974). Although these results imply that lead affects only CMI functions, humoral defects have also been observed. Koller and Kovack (1974) demonstrated that exposure to lead resulted in depressed primary and secondary responses when monitored by plaque-forming (Jerne-type) assays of antibody production. Koller et al. (1976) suggested that these results were due either to a direct effect of lead upon plasma cell antibody synthesis or to a dysfunction in T-/B-memory cell ability to maintain antigenic specificity.

The effects of cadmium exposure upon immune function are less well-defined. Generally, the immunotoxic effects of cadmium are similar to those of lead: decreased resistance to endotoxin, bacterial, and viral challenges (Cook et al., 1974; Gainer, 1977) and decreased plaque responses and antibody titers in rodents (Koller, 1973; Koller et al., 1975; Graham et al., 1978). However, some have observed increased resistance to encephalomyocarditis virus in cadmium-treated hosts (Exon et al., 1979), while other studies have shown that the effects of cadmium on antibody titers are dependent upon the time of exposure relative to the point at which the host received the test

antigen (Jones et al., 1971). Unlike lead, cadmium appears to augment rather than impede the memory response of lymphocytes (Koller and Roan, 1980).

The effects of cadmium on macrophages are even less clear. Koller and Roan (1977) reported that cadmium exposure promoted phagocytosis, yet it was also shown that the metal treatment impaired EA-rosette formation (an index of antibody F_c receptor activity; Hadley et al., 1977), was directly toxic to these cells (Loose et al., 1978b), and depressed total phagocytic capacity (Loose et al., 1978a). Thus, the only conclusion agreed upon by many researchers is that there is a depression, by uncertain mechanisms, of humoral responses following cadmium exposure.

Mercury, in both the organic and inorganic forms, was of particular interest to the immunotoxicologists since the metal will accumulate in the major organs of the immune system: liver, spleen, and thymus. Interestingly, the age of the host had an important influence on the immunotoxic potential of mercury (Ohi et al., 1976; Koller et al., 1977). Both forms of the metal can result in the decreased resistance to viral lethality, latency to tumor detection, and plaque-forming responses (Koller, 1973; Gainer, 1977). While inorganic forms of mercury can block lymphoproliferative responses (Gaworski and Sharma, 1978), the organic form (methylmercury) cannot block the response when the B-cell mitogen lipopolysaccharide is used (Koller and Roan, 1980). However, methylmercury can block proliferation when

the mitogens concanavalin A or phytohemagglutinin are employed (Hirokawa and Hayashi, 1980). These results are in agreement with the observation of thymic cortex atrophy that results following subchronic mercury exposure (Blakely et al., 1980). The various observations reported make defining a mechanism for mercury immunotoxicity difficult, but it appears that the lymphocytes are the primary targets, since mercury has no apparent effect on macrophage phagocytic activity or that of the antibody F_c receptors (Koller et al., 1980).

Other transition elements to which man may be exposed directly in industrial settings or indirectly by consumption of treated foods include aresenic, tin, nickel, and cobalt. Still other metals, such as iron, copper, selenium, and zinc display immunomodulating effects when the level of exposure is great or there is a deficiency in intake. Metals that only recently have been receiving greater scrutiny due to occupational exposure risks include vanadium, chromium, titanium, gallium, and germanium (Plunkett, 1976; Caren, 1981).

In studies with tin, lymphoid depletion and antibody suppression were observed in rats after long- or short-term exposure (Verschuuren et al., 1970; Hioe and Jones, 1984). Of note, the degree of thymic atrophy attained was dependent upon the alkyl chain length of the ligand, and these observed toxic responses were unique to rats (Seinen and Penninks, 1979). In vitro, organotin complexes are highly

toxic to thymocytes, and in vivo these results are reproduced but without any associated myelotoxicity or effects upon non-lymphoidal tissues. Resulting CMI functions, such as delayed hypersensitivity, graft vs host response, and lymphoproliferation due to T-cell mitogens are all decreased after host exposure. Humor-al responses such as plaque formation are reduced secondary to the effect on T-cells (Dimitrov, 1981), while macrophage activities remain unchanged (Seinen et al., 1977).

Arsenicals are commonly encountered in herbicides, rodenticides, insecticides, and as antiviral agents/growth promoters in livestock feed (Gainer and Pry, 1972). In 1918, Toyama and Kolmer showed that low levels of arsenic exposure enhanced antibody production, while at high levels, suppression was the result. Gainer (1972) noticed that mice treated with high levels of arsenic had decreased viral resistance. As with the studies with lead, it was shown that the effect was the result of impaired interferon synthesis/release. Fowler (1978) noted that arsenic toxicity was due to binding to enzyme sufhydryl groups and to nucleic acids in RNA. This would result in lowered production of interferon as well as antibodies (Blakely et al., 1980). The primary effect of arsenic appears to be upon the humoral response as most CMI activities remained unaffected (Kerkvliet et al., 1980).

Exposure to nickel compounds is primarily an occupational hazard. Workers exposed to nickel-containing

dusts display increased incidences of respiratory infections (Sunderman, 1977). Research focused upon the effects of nickel on alveolar macrophage function, with *in vivo* studies resulting in enhanced phagocytic activity but also evidence of alveolar lipoproteinosis (Jarstrand et al., 1978). When studies were performed *in vitro*, few cytological alterations were observed (Camner et al., 1978). Graham et al. (1975) concluded that there was not a direct effect of nickel upon alveolar macrophage lipid metabolism, but that surrounding pneumocytes increased their secretion of lipids which were then affixed to the leukocyte surface. Although inhibition of macrophage ATPase by nickel resulted in decreased phagocytic activity (Graham et al., 1978), others claimed that nickel exposure enhanced macrophage activity. While exposure to the metal resulted in decreased resistance to viral and bacterial challenges (Gainer, 1977; Adkins et al., 1979), Treagan and Furst (1970) demonstrated that these responses were due to defects in interferon production via blockage of mRNA formation. Figoni and Treagan (1975) also showed that nickel treatment resulted in lowered antibody titers as the result of the binding of the metal to DNA (to block transcription) or to B-cell membranes which then blocked interactions with macrophages and T-helper cells.

With the essential metals, iron, selenium and zinc, the primary emphasis has been on the immunological effects from deficiency rather than from excessive exposure. Iron excess has been shown to decrease the bactericidal action of

leukocytes and to inhibit chemotaxis, but without altering immunoglobulin synthesis, complement fixation, or antigen opsonization by serum factors (Payne and Finkelstein, 1978; Weinberg, 1978). Iron deficiency can impair leukocytic antibacterial activity, decrease T-lymphocyte transformation, and depress immunoglobulin synthesis. However, many of these effects are believed to be secondary to other hematological disturbances resulting from iron deficiency (Koller, 1980).

Selenium deficiency resulted in lowered antibody titers in newly-vaccinated hosts (Sheffy and Schultz, 1978), and it was suggested that a balance be maintained between selenium and vitamin E levels in order to maintain proper immunoresponsiveness (Sheffy and Schultz, 1979). When used as a supplement, selenium can potentiate the effects of a test vaccine (Desowitz and Barnwell, 1980) and enhance primary and secondary immune responses while ameliorating depressed antibody titers which resulted from host exposure to other toxicants (Spallholz et al., 1975; Koller et al., 1979).

The route of exposure to excess zinc affects the host immune response. After oral administration, no alteration in immune function was detected in several different animal models (Gainer, 1977; Hill, 1979). When zinc was given intraperitoneally, resistance to bacterial challenge varied depending upon organism tested (Sobocinski et al., 1977). The effects from zinc deficiency are more straightforward; thymic atrophy, decreased antibody synthesis as the result

of diminished helper T-cell function (Fraker et al., 1977; Luecke et al., 1978; Fernandes et al., 1979), and overall reduced immunoresponsiveness secondary to lowered serum corticosterone levels (Jardieu and Fraker, 1979). Mitogenic responsiveness appears to be unaffected by either a zinc-deficient or zinc-excessive state (Mulhern, 1980). A narrow range of zinc levels appears to be required for optimal immune activity. Although the role of zinc in the mechanism of proper immune function is becoming clearer, the macrophage has been identified as one component which readily uses zinc for both structural maintenance and self-reparation (via metallothionein apoprotein role in the detoxification of oxygen radicals) (Chvapil, 1973; Patierno et al., 1983).

In all the studies of the immunotoxicity of metals, variables such as the type of metal complex, the metal valence, the animal model, the length and route of exposure, nutritional status, and whether in vivo or in vitro assay systems are used must be taken into account when interpreting the data to form the immunotoxicological profile. The studies with nickel and with zinc highlight the pitfalls in assuming that only one series of studies can possibly be used to define the immunomodulating potential of any test metal.

Glucose-6-Phosphate Dehydrogenase and Glutathione Reductase

The hexose monophosphate shunt, also known as the pentose phosphate or phosphogluconate pathway, is a mechanism available to cells for obtaining energy from the oxidation of glucose. Its functional role in most cells is to generate reducing equivalents (NADPH) in the extra-mitochondrial cytoplasm, to convert hexoses into pentoses to be used in the synthesis of nucleic acids, and to convert pentoses to heptoses (via transketolase and transaldolase reactions) and ultimately to hexoses for use in glycolysis (Lehninger, 1979). In cells which reside in oxygen-poor environments, such as the peritoneal macrophage, both the shunt and enhanced glycolysis are the principal sources of energy for phagocytosis (Axline, 1970; Karnovsky et al., 1970; Rossi et al., 1975).

Glucose-6-phosphate dehydrogenase (G6PDH or Zwischenferment) is the first enzyme of the hexose monophosphate shunt. It catalyzes the oxidation of D-glucose-6-phosphate to a D-gluconolactone-6-phosphate intermediate with the subsequent release of NADH or NADPH (+ H^+). Although initially discovered in erythrocytes, G6PDH has since been isolated and purified from a wide variety of animals, plants, and microorganisms (Pon, 1964). This has allowed for detailed studies of the structural, regulatory, and catalytic activities of the enzyme.

The enzyme isolates from animal, plant, or microbial sources display a wide variety of characteristics. Certain

bacteria, such as Pseudomonodaceae, possess separate forms of G6PDH for use in the hexose shunt and for fermentation. In other organisms (i.e. Leuconostoc mesenteroides), one form of G6PDH serves both pathways with the enzyme displaying a dual-nucleotide specificity (DeMoss et al., 1953). Even within a family, such as the Saccharomyces yeasts, the enzymes from S. carlsbergensis and from S. cerevisiae are known to have differing amino acid compositions and to utilize different kinetic mechanisms. Still, researchers often interpret their results with respect to the enyzme source with which they are not actually working (Kuby et al., 1974; Levy, 1979). The isolation of the G6PDH from these varied sources also points out the heterogeneity of enzymes; in general, G6PDH from animal and fungal sources are more labile than that from bacterial sources. Through the use of exogenous agents such as glycerol, $NADP^+$, or glucose-6-phosphate (Malcolm and Shepherd, 1972), the enzyme may be stabilized during isolation and purification but this results in altered activities.

Although G6PDH from many sources has been found in the form of non-active monomeric units, the dimer is the catalytically active form in animal G6PDH (Yoshida and Hoagland, 1970). In studies of the yeast G6PDHs, it was found that the active form was not the dimer but a tetramer (Robbins et al., 1975). Though certain forms of G6PDH may be isolated as larger oligomers and still maintain catalytic

activity, the yeast enzyme appears to be restricted to nothing larger than the tetramer (Yue et al., 1969; Kuby et al., 1974). These studies indicated that the presence of $NADP^+$ was needed to promote tetramer formation. Cohen and Rosemeyer (1969), using an erythrocyte model, concluded that ionic bonding stabilized interdimer associations within the tetramer, whereas hydrophobic forces stabilized intradimer interactions. These latter forces were also described by Yue et al. (1969), but they claimed that the role of ionic forces was minimal. It was shown that $NADP^+$ stabilizes the enzyme, but upon reduction to NADPH or as a result of enzyme modification, the G6PDH is inactivated with subsequent dissociation to the inactive monomeric units (Yoshida, 1966; Bonsignore et al., 1968). The latter authors claimed that this effect was unique to erythrocyte G6PDH (i.e. "structural $NADP^+$"), but Yue et al. (1969) reported similar findings in yeast. The $NADP^+$, rather than stabilizing the tetramer by interacting directly with the subunits, modified the solvent-cage effect by displacing water molecules and enhancing the attractive forces between the dimers. This association of dimers leads to the formation of an active tetrahedral structure within a solvent sphere.

In studies of substrate specificity, the majority of G6PDHs require that the beta-D-glucopyranose-6-phosphate form be present (Salas et al., 1965). The authors noted that anomeric specificity might constitute a rate-limiting factor, but they determined that cellular phosphoglucose

isomerase and spontaneous anomerization of the alpha form would alleviate this possible effect. Although all G6PDHs can utilize structurally similar substrates (i.e ribose-5-phosphate, 2-deoxyglucose-6-phosphate) the highest V_{max} and lowest K_m are achieved with glucose-6-phosphate (K_m = 0.051 mM: Yoshida, 1967). Extensive modifications of the substrate (i.e. at the anomeric carbon or methylation of the 2-deoxy form) result in a loss of enzymatic activity (Egyud and Whelan, 1963; Yoshida, 1966).

Reaction measurements using glucose indicated that in the presence of certain anions such as phosphate, sulfate, or bicarbonate, the activity of G6PDH was increased. However, when these anions were present with glucose-6-phosphate substrate, the activity was inhibited (Anderson and Nordlie, 1968). Following studies with ATP and other nucleoside phosphates, this group concluded that the C-6 phosphate, the nucleotides, and the select anions shared a common binding site in the active enzyme.

Five classes of G6PDH have been defined regarding coenzyme specificity: $NADP^+$-specific, $NADP^+$-preferring, dual nucleotide specificity, NAD^+-preferring, and NAD^+-specific. The majority of G6PDHs belong to the first three groups; far fewer utilize NAD^+ as the cofactor of choice. Although S. carlsbergensis is known to be $NADP^+$-specific (Levy, 1979), only recently has S. cerevisiae been shown to act in a similar manner (Cohen et al., 1987) although a K_m value

(0.0075 mM) has been previously reported (Adams et al., 1976). In addition to specificity for substrate and cofactor, divalent magnesium (Mg^{2+}) is needed for proper G6PDH function (Kornberg, 1950).

Initial investigations of the kinetic mechanism by Kuby et al. (1974) suggested a rapid-equilibrium random model for yeast; $NADP^+$ and glucose-6-phosphate bind the enzyme independently, with the rate-limiting step being the interconversion of ternary complexes. Schachet and Squire (1976) also proposed this random mechanism for bovine adrenal G6PDH, but their experimental Dixon analyses obtained using an NADPH inhibitor showed competitive inhibition against $NADP^+$ and noncompetitive inhibition against the sugar phosphate, indicative of an ordered sequential mechanism. Olive et al. (1971) proved conclusively that the mechanism was ordered sequential ("Ordered Bi Bi") whereby $NADP^+$ is bound first to the enzyme and the product NADPH is released last. This mechanism has since been identified in G6PDH from several sources, such as human erythrocytes (Yoshida, 1973), porcine (Kanji et al., 1976b) and rat liver (Thompson et al., 1976).

Although $NADP^+$ has been shown to be required for proper tertiary/quarternary structure and enzyme activity, and the reduced NADPH has been demonstrated to be an effective inhibitor of G6PDH, it is the ratio of these two forms which plays an important role in regulating enzyme activity in vivo (Yoshida, 1973; Yoshida and Lin, 1973). Polakis and

Bartley (1966) reported that the $NADPH/NADP^+$ ratio in S. cerevisiae was near unity. Studies with yeasts (Passonneau et al., 1966; Kuby et al., 1974) indicated that NADPH could act as a potent inhibitor, but a regulatory role was not investigated; Grove et al. (1976) suggested that NADPH was unlikely to have any regulatory role.

In inhibition studies with NADPH and rat liver G6PDH, Eggleston and Krebs (1974) concluded that a de-inhibition mechanism to counterbalance the NADPH inhibition was present in intact cells. Oxidized glutathione (GSSG) was suggested to be the de-inhibiting agent in both liver and erythrocytes. This was the logical conclusion, since in the cell the levels of reduced glutathione (GSH) are maintained by glutathione reductase, a cytoplasmic enzyme which readily utilizes the G6PDH/hexose monophosphate shunt-generated NADPH. The removal of the inhibitory NADPH thermodynamically and stoichiometrically drives the G6PDH reaction toward product formation and insures that maximal enzyme activity in vivo is dependent upon the availability of substrate and cofactor only.

Glutathione reductase (GSSG-R, EC 1.6.4.2) catalyzes the reduction of oxidized glutathione (GSSG) to glutathione (GSH) using NADPH. In many cells, the ratio of GSH:GSSG is adequately high (Gunthenberg and Rost, 1966) and the enzymatic conversion is limited only by the availability of substrate. This cytoplasmic enzyme is the second enzyme of the glutathione redox cycle (glutathione peroxidase [GSHPX]

is the other) and is responsible for regenerating the reduced glutathione substrate for GSHPX. In peritoneal macrophages, GSSG-R is also a controlling force in the activity of the hexose monophosphate shunt by regulating the production and release of $NADP^+$ (Strauss et al., 1969). Therefore, cellular GSSG-R is both in control of and controlled by the hexose shunt and any effects upon its activity.

The majority of studies have dealt with the activity of GSSG-R in erythrocytes (Massey and Williams, 1965; Icen, 1967). Maintenance of reduced glutathione levels is required to provide a source of sulfhydryl integrity (Allen and Jandl, 1961; Jacob and Jandl, 1962) and prevent hemolysis. Inborn errors in GSSG-R activity often result in leucopenia, thrombocytopenia, and chronic nonspherocytic hemolytic anemia (Carson et al., 1961) and increased susceptibility to many toxicants.

Glutathione reductase belongs to the class of enzymes that contain functional disulfides in addition to a flavin coenzyme (FAD) as part of the catalytic site; others include thioredoxin reductase and lipoamide dehydrogenase (Douglas, 1987). Each of these flavoproteins consists of two nearly identical polypeptide chains, each with a reactive cystine and two molecules of FAD. Among the enzymes cited, there is virtually no cross-reactivity due to the specificity for disulfide substrates (Williams, 1976). The action of GSSG-R on mixed disulfides that contained at least one-half

glutathione or gamma-glutamylcysteine has been reported (Woodin and Siegel, 1968). Of all the analogs assayed, only a selenium-diglutathione (G-S-Se-S-G) compound resulted in GSSG-R activity greater than 20% of the levels obtained with GSSG (Ganther, 1971). Rat liver GSSG-R has also been shown to catalyze the reduction of a glutathione-Coenzyme A mixed disulfide (Ondarza et al., 1974). When further analyzed, it was determined that the catalyzing enzyme was distinct from GSSG-R (Ondarza, 1976). Although this dual effect has also been observed in yeast, no second enzyme has been isolated (Ondarza and Martinez, 1966; Eriksson et al., 1974).

With respect to the pyridine nucleotide cofactors, the lipoamide enzyme is NADH-preferring and the thioredoxin protein is NADPH-preferring. Yeast and erythrocyte GSSG-R are NADPH-specific (Staal and Veeger, 1969), although some preparations have been shown in vitro to have activity, albeit highly reduced, in the presence of NADH (Icen, 1967).

The catalytic center of the GSSG-R protein contains a molecule of FAD in the vicinity of a disulfide bridge. The enzyme will accept two electrons from NADPH and eventually transfer two electrons to the GSSG substrate thereby reducing it to two molecules of GSH. The NADPH binds in the region of the active site to form an enzyme-oxidized nucleotide complex (Williams, 1976). The first electron is picked up by the distal sulfur atom to yield a thiol moiety and a proximal S^- (thiolate) group: at this point the enzyme is designated as half-reduced (EH_2) (Massey and Williams,

1965). The active site is energetically dynamic, such that the extra electron on the thiolate ion is free to transfer to the bound FAD molecule. The complexity of the reaction has led to various descriptions of the active site at this stage: a biradical (Searls et al., 1961), a charge-transfer complex (Massey and Ghisla, 1974), or a covalent FAD-S bond site (Palmer and Massey, 1968).

The flavin molecule is tightly bound to the active site (Massey and Williams, 1965), with two molecules per dimer. The flavin is located within a hydrophobic region that Burleigh and Williams (1972) determined was created by a tight-loop structure that resulted from the presence of half-cystine residues in the peptide chain. Such looping would allow for multiple weak force (i.e. van der Waals) interactions and for positioning of the FAD close to the disulfide moiety to allow for electron sharing.

After the first thiol group is generated, the oxidized nucleotide cofactor is displaced. Subsequently a molecule of GSSG undergoes a thiol exchange at the distal sulfur atom to yield a mixed disulfide (E-S-S-G) and the first molecule of GSH. The proximal thiolate reaccepts the electron previously donated to the FAD, and then displaces the second GSH molecule via a nucleophilic attack (Bulger and Brandt, 1971). If a second molecule of NADPH binds soon after the first nucleotide dissociates and before the active site can be regenerated through the reaction with GSSG, a four electron-reduced enzyme (EH_4) containing reduced FAD ($FADH_2$)

and vicinal thiols will form resulting in a catalytically inactive enzyme.

A more detailed reaction mechanism (Figure 2) based on a biophysical chemical analysis of intraprotein bond angles and lengths, spectrophotometric analysis of intermediary structures, stopped-flow kinetic analysis, and X-ray crystallographic studies has recently been suggested (Pai and Schulz, 1983; Douglas, 1987). Briefly, the highlighted states are similar to those described above: NADPH binds to the oxidized enzyme (State 1) leading to cleavage of the redox-active disulfide and $NADP^+$ formation (State 2). Because of structural constraints in the protein and the location of the NADPH molecule on the opposite side of the FAD, Douglas (1987) suggested that without moving the flavin ring, the process of electron transfer must be mediated transiently through FAD (but at a rate much faster than that needed for the structural rearrangement of the flavin during conversion from oxidized to reduced and back to an oxidized state). The GSSG molecule is then bound (State 3) at the distal thiol group (State 4) with the initial GSH release followed by the subsequent nucleophilic displacement of the second GSH molecule (State 5) such that the active disulfide and enzyme are regenerated.

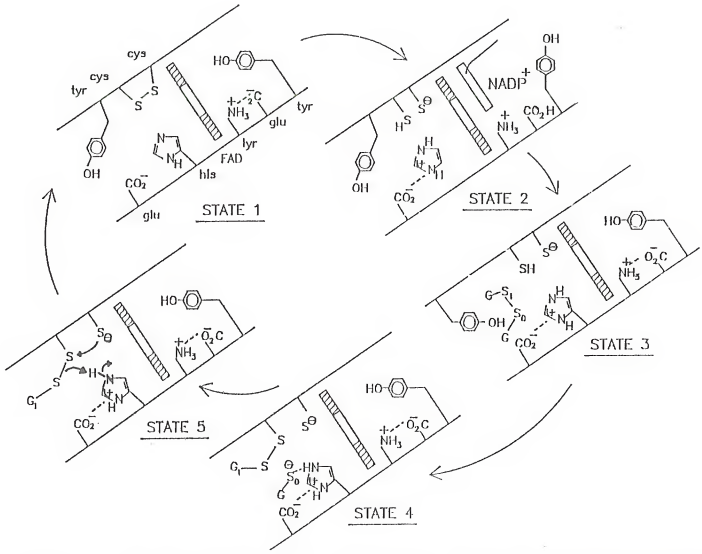


Figure 2. Revised mechanism of glutathione reductase catalytic activity.

In determining the kinetic mechanism for GSSG-R, Bulger and Brandt (1971) suggested that another intermediate, EH_2-NADPH, could be formed. If the levels of GSSG are low, this can then proceed to form the EH_4 complex; if NADase is present, the second nucleotide can be cleaved to regenerate the active EH_2 structure (Massey and Williams, 1965). By monitoring the inhibition of activity by using $NADP^+$ (competitive against NADPH, noncompetitive against GSSG), instead of a hypothesized binary complex mechanism ("Ping Pong Bi Bi"), GSSG-R was found to follow a mixed, ordered sequential pattern ("Ordered Bi Bi") (Mannervik, 1969). A determination of K_m values for both GSSG and NADPH is complicated by the effects of reaction ionic strength upon enzyme activity (Icen, 1967; Staal and Veeger, 1969). However, in erythrocytes, K_m values of 0.010-0.013 mM and 0.019-0.125 mM for NADPH and GSSG, respectively, have been calculated in systems with ionic strengths of 0.03-0.30 M (Scott et al., 1963; Staal and Veeger, 1969). With yeast GSSG-R, K_m values of 0.004 and 0.055 mM, respectively, have been calculated (Massey and Williams, 1965); however, the exact kinetic mechanism will shift between the sequential mechanism and the binary complex pattern dependent upon the concentration of GSSG present.

With a clearer picture of the active site mechanism of both the dehydrogenase and the reductase, more precise descriptions of the inhibitory effects of many metal complexes have been published (Icen, 1967; Anderson et al.,

1968). In these systems, inhibition resulted from the binding of metals at the site designated for the substrate, or from binding to the enzyme such that the regeneration of the active site was affected. Although many *in vitro* studies are able to report the levels of inhibition when metals are added to purified enzyme systems, the concentrations of inhibitor as well as substrates are often in excess of physiological levels. Nonetheless, it has been widely accepted that such studies serve only to provide plausible mechanisms of inhibition since in the intact cell the effects of pH and intracellular binding can dramatically affect levels of free metal.

Macrophage (Peritoneal and General) Functional Biochemistry

The primary function of the macrophage is to engulf and process exogenous agents for presentation to T-lymphocytes in the initiation of an immune response, or to act as direct killers of host-invading pathogens. The enzymology involved in both the phagocytic and killing phases has been well studied in polymorphonuclear leukocytes (PMN) such as neutrophils (Sbarra et al., 1971 and 1972; Gabig and Babior, 1981), but less so in macrophages (Axline, 1970; Romeo et al., 1973; Karnovsky et al., 1975a, 1975b). The enzymes G6PDH, GSSG-R, and GSHPX each have a role crucial to the ability of the macrophage to phagocytize antigen and to utilize oxygen effectively in the killing of trapped microorganisms.

During phagocytosis, there is an increase in cellular respiratory activity, glycolysis, and oxidation of glucose through the hexose monophosphate shunt (HMS) (Becker et al., 1958; Sbarra and Karnovsky, 1959; Myrvik and Evans, 1967) in a process known as the respiratory burst. These increased activities provide the energy required for phagocytosis (Karnovsky, 1962) as well as the oxygen (O_2) ultimately used for intracellular killing. Several potent oxygen metabolites including superoxide anion ($O_2^{\cdot-}$), perhydroxy and hydroxyl radicals ($HO_2^{\cdot}$ and $^{\cdot}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) are produced within the macrophage (Klebanoff, 1980b). As outlined in Figure 3, the process requires the participation of G6PDH in the initial formation of hydrogen peroxide as well as its ultimate removal from the cell via the glutathione redox system.

The production of the superoxide generates oxidized cofactor, $NADP^+$, as a product. Zatti and Rossi (1965) noted that in macrophages, there was an increase in the ratio of $NADP^+$ to NADPH within 1 min after initiation of phagocytosis Since $NADP^+$ acts as the rate limiting substrate for the HMS pathway, its regeneration via NADPH oxidase may account in part for the increased respiratory activity observed (Sbarra et al., 1971). The early increase in $NADP^+$ levels is believed to arise mainly from the activation of cytoplasmic GSSG-R. The reductase is activated within 15 seconds after antigen contact with the outer membrane (Strauss et al., 1969).

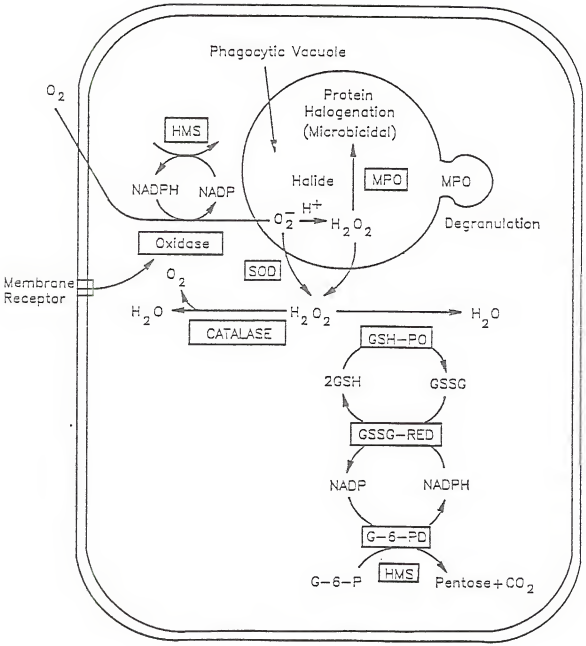


Figure 3. Macrophage mechanism of intracellular killing and the biochemistry of oxygen activation and subsequent detoxification.

As intraphagosomal levels of superoxide and perhydroxy radical increase, free oxygen and hydrogen peroxide are produced either by spontaneous dismutation or by the activity of superoxide dismutase (SOD). The intraphagosomal hydrogen peroxide in neutrophils and eosinophils is utilized by myeloperoxidase (MPO) to produce activated halides for killing (Klebanoff, 1967 and 1968). Although there is very little MPO in mouse peritoneal macrophages (PEM) (van Furth et al., 1970), in other rodents MPO has been detected in the macrophage nuclear envelope, the cisternae of rough endoplasmic reticuli, and in Golgi bodies (Cotran and Litt, 1970; Robbins et al., 1971). Therefore, mouse PEM function like MPO-deficient neutrophils and kill ingested targets primarily through toxic oxygen metabolites.

The mechanism for removal of excess hydrogen peroxide in most leukocytes is via catalase, a reaction that consumes two molecules of peroxide to yield oxygen and water. While there is abundant catalase in alveolar macrophages (Gee et al., 1971), the level is low in mouse PEM (Simmons and Karnovsky, 1973). The hydrogen peroxide produced in the phagolysosome can seep out and enter the cytoplasm so that without sufficient catalase, there could be the production of hydroxyl radicals as well as peroxidative damage to intracellular membranes. With low levels of catalase, the GSHPX system is the primary mechanism of peroxide detoxification within mouse PEM. In this system, hydrogen peroxide is converted to 2 molecules of water at the cost of 2

glutathione molecules (GSH). The oxidized glutathione product (GSSG) is reduced back to glutathione by GSSG-R which utilizes a molecule of NADPH formed in G6PDH/HMS reactions.

The glutathione-dependent detoxification system is most effective with lower levels of peroxide (Nicholls and Schonbaum, 1963). In mouse PEM, it has an enhanced capacity due to the deficiency of other enzymatic detoxification mechanisms (i.e. catalase). A loss in the activity of the peroxidase results in the buildup of peroxides and other oxygen products which damage the cell and decrease cellular viability. A major effect from a build up of peroxides is a decrease in the overall length and strength of the respiratory burst with a shift from a normal 20 min burst after ingestion to a period of only 3-5 min (Roos et al., 1979).

Though its primary role in the macrophage is to serve as a substrate for the peroxidase cycle, reduced glutathione protects intracellular macromolecules from attack by conjugating with electrophilic compounds (Al-Turk et al., 1987). Glutathione is also used to prevent the formation of hydroxyl radicals when SOD activity is low or when levels of superoxide anion are high. While the glutathione redox cycle removes hydrogen peroxide to prevent its reaction with superoxide anion, the reduced tripeptide may directly block oxygen radical formation by chelating cellular iron which is used in the Haber-Weiss reaction (Haber and Weiss, 1934; Perchellet et al., 1986). Not only is the presence of

reduced glutathione in the cells crucial to overall cellular maintenance, but a balance between the levels of the oxidized and reduced forms is critical to maintaining optimal enzyme activity without inducing feedback inhibition of enzymes involved in the redox cycle.

Many metals including bismuth, cadmium, gold, mercury, nickel, silicon, tin, and titanium have been studied for their effects on the viability and antimicrobial/phagocytic activities of murine PEM (Loose et al., 1978a, 1978b; Soutar and Coghill, 1984, Tam and Hinsdill, 1984; Richards et al., 1985; Fisher et al., 1986; Zimmerman et al., 1986). Other studies have determined the effects of metal on the oxidative burst based upon the chemiluminescence of luminol or lucigenin following ingestion of zymosan particles (Allen and Loose, 1976; Parnham et al., 1983; Moller-Madsen et al., 1986) or upon macrophage oxygen uptake (Cross et al., 1970; Loose et al., 1977). Other studies have focused on the interaction of the metal with structural components such as microtubules (Allison et al., 1971; Ryter, 1985), membrane receptors (Cook et al., 1984a, 1984b), or ionophores (Stickle et al., 1984) as factors in the modulation of macrophage activity. Little work has been performed to study the effects of metals on the enzymes involved in the respiratory burst or in the production and/or removal of oxygen metabolites.

Anti-listerial/-bacterial Activity of Peritoneal Macrophages

The bactericidal and/or growth inhibitory (bacteriostatic) action of phagocytic cells on ingested microorganisms serves as an important defense mechanism of the host against bacterial infection. The accumulation and mobilization of mononuclear phagocytes to foci of infection is critical to prevent fulminating growth of the bacteria before adequate levels of T-effector cells are present and macrophages can be activated (North, 1970). The immune response to bacterial infection has been well studied *in vivo* and *in vitro* (Mackaness, 1962; Simon and Sheagren, 1971; Pearson and Osebold, 1973) and indicates that the cellular reactions in the killing of bacteria involve both lymphocytes and macrophages.

Listeria monocytogenes serves as an ideal organism for the study of the CMI system in response to a bacterial infection. The role of the humoral system in resistance to this pathogen has been shown to be of little significance; antibodies to *Listeria* are not produced in significant quantities during a primary infection (Mackaness, 1962; North and Deissler, 1975) nor is resistance passively transferrable within a species via serum (Miki and Mackaness, 1964). The transfer of immunity is only possible through monocytes/macrophages and other lymphoid cells from hosts preimmunized with *Listeria* ("*Listeria*-immune") (Mackaness, 1969). Exposure to *Listeria monocytogenes* results in an acute, potentially lethal infection in mice, with the degree

of resistance among strains being genetically controlled by a single dominant, non H-2 linked autosomal gene designated Lr for Listeria resistance (Cheers and McKenzie, 1978; Skamene et al., 1979; Stevenson et al., 1981).

The virulence of gram-positive Listeria monocytogenes appears to be dependent upon its successful parasitization of cells from the mononuclear phagocyte system. Listeria possesses a surface sufficiently attractive to phagocytes so that it need not be opsonized to be engulfed (Harrington-Fowler et al., 1981). Though Listeria may attach directly to the macrophage and stimulate phagocytosis, serum factors such as complement component C3b may serve as phagocytosis-enhancing opsonins (Baker et al., 1977).

Following attachment to the resident macrophage surface, Listeria is internalized and encapsulated in an organelle known as the phagosome. The phagosomes are designated as "loose" in that they are generally large, capable of fusing with lysosomes, and can accept lysosomal contents upon degranulation (North and Mackaness, 1963a, 1963b). Unlike intracellular pathogens such as Mycobacterium tuberculosis and Brucella melitensis which are encapsulated in "tight" phagosomes and are able to block phagolysosomal fusion and degranulation (Fauve and Delaunay, 1966), Listeria are capable of causing a delay in the fusion-degranulation process (de Heer et al., 1980). Although fusion eventually occurs, Pesanti (1978) and de Heer et al. (1980) concluded that the intracellular survival

of Listeria was dependent on resistance to, and not evasion of, lysosomal enzymes. Although several other pathogens multiply in macrophages, such as Mycobacterium lepraemurium (D'Arcy Hart et al., 1972), Salmonella typhimurium (Carrol et al., 1979), and Toxoplasma gondii (Jones and Hirsch, 1972), only the latter has been shown to have a direct correlation between its intracellular demise and phagolysosome fusion.

Apparently, resident macrophages are not fully capable of mounting an adequate killing effort against Listeria in the early stages of infection. Macrophages from Listeria-immune animals are structurally different from the resident cells obtained from untreated mice (North and Mackaness, 1963a, 1963b) and display enhanced bactericidal activities. After processing of digested Listeria occurs, CMI pathways can be activated so that lymphokine-mediated macrophage activation occurs. This results in a macrophage population with enhanced killing capacities associated with increased metabolic activity and toxic oxygen metabolite production (Ratzan et al., 1972; Johnston, 1978).

During the period prior to macrophage activation, Listeria is capable of reproducing within the phagosome. In cultured resident macrophages, Listeria replication occurs within a short time, with an estimated generation time of 90 min (Bennedsen et al., 1977; Harrington-Fowler et al., 1981). An assessment of the fate of intracellular Listeria is complicated by the continued phagocytizing of the

bacteria by cells which also contain multiplying organisms. When levels of intracellular replication are quite high, derangements of the macrophage cytoplasm occur leading to cell death and lysis and subsequent release of virulent progeny (Armstrong and Sword, 1964).

Although control of the initial bacterial infection is ascribed to macrophages in general, the peritoneal exudate population is composed of several subpopulations each with its own bacteriostatic/bactericidal activity (Harrington-Fowler and Wilder, 1982). Within a species, different breeds reflect genetic differences in subpopulation phagocytic and killing capacities (Zembala and Asherson, 1970), distribution of Ia (immune response antigen) molecules needed for antigen interaction with T-cells to promote the enhanced cellular immune response (Cowing et al., 1978) and, responsiveness to lymphokines for activation (Lee et al., 1981). The functional heterogeneity of the PEM subpopulations is reflected in the observation that for a given bacterial sample several organisms may be killed after phagocytosis while others survive and multiply (Jenkin and Benacerraf, 1960).

Several studies have been performed to clarify the fate of intracellular Listeria (Wilder and Edberg, 1973; Harrington-Fowler et al., 1981; Harrington-Fowler and Wilder, 1982; Godfrey et al., 1983). The majority of studies with mouse PEM have employed cells from Listeria-immune hosts or cells in an activated state due to host

pretreatment with thioglycate, BCG (Bacille Calmette-Guerin), caseinate or heat-killed Listeria. The latter study indicated that listericidal activity of these PEM was strong during the first 17 hr post-infection of a PEM monolayer (Godfrey and Wilder, 1984).

Fewer studies have evaluated the fate of Listeria in resident cells. The in vitro study of Godfrey et al. (1983) demonstrated significant bactericidal activity by resident macrophages during the first 3 hr post-infection, after which bacterial growth resumed. The killing systems inherent to these cells appeared to be only partially effective against facultative intracellular pathogens such as Listeria, and these systems were rapidly exhausted during an active infection. The early killing may also have been the result of the action of a specific bactericidal subpopulation, which with time was overwhelmed by the continuous replication within weak or nonfunctional subsets. In addition to the different subpopulation antimicrobial activities, the susceptibility of Listeria to destruction was related to inherent bacterial factors associated with growth characteristics.

Unlike the mechanisms that utilize activated oxygen species for the intracellular killing of Toxoplasma, Leishmania and Candida (Murray and Cohn, 1980; Sasada and Johnston, 1980; Murray, 1981), the precise mechanism by which resident and activated macrophages kill Listeria is not clear. The cited organisms give rise to increases in

intracellular superoxide anion and hydrogen peroxide production following the phagocytic stimulus (Bryant et al., 1982; Badwey et al., 1983). However, activated and resident macrophage monolayers display no such increase when presented with opsonized Listeria (Godfrey and Wilder, 1984; Hashimoto et al., 1986). The addition of oxygen scavengers or inhibitors of enzymes involved in oxygen-dependent antimicrobial activity, such as catalase or SOD, had no impact on bactericidal activity (Czuprynski et al., 1983). When catalase is employed as a scavenger, only slight decreases in bacterial killing occur and this is attributed to an accessorial role for hydrogen peroxide in the ultimate destruction of Listeria previously damaged by non-oxidative mechanisms (Shultz and Wilder, 1971; Czuprynski and Balish, 1981).

The apparent nonresponsiveness of the macrophage to increase the levels of activated oxygen species after Listeria ingestion has been implicated as a major factor in the virulence of this organism (Wilson et al., 1980). That resident and activated cells are not able to produce high levels of reactive oxygen species in response to Listeria seems to be a factor related to the bacteria rather than to the phagocyte. A comparison of the oxidative metabolism with that of macrophages exposed to Mycobacterium intracellulare shows significantly lower levels of hydrogen peroxide release and chemiluminescence after phorbol myristate acetate treatment (Saito et al., 1986). Listeria

itself has been shown to possess the capacity to produce and detoxify superoxide and hydrogen peroxide (Godfrey and Wilder, 1984). It is likely that the macrophages exhibit increased production of toxic oxygen metabolites but these are subsequently neutralized by bacterial enzyme systems.

Although in in vitro studies resident macrophages are as capable of limited killing of Listeria as activated cells from immune-boosted mice (Spitalny, 1981), cells harvested after 24 hr from an infected host have greatly increased bactericidal activities (Czuprynski et al., 1984). The latter exudate is actually a heterogeneous population of resident PEM, newly arrived monocytes and neutrophils. The monocytes and neutrophils both exhibit greater levels of activated oxygen metabolite production and are more listericidal than the activated macrophages (Czuprynski et al., 1983; Lepay et al., 1985). Preventing monocyte recruitment to the site of infection results in a failure of the host to resist a Listeria challenge (Mitsuyama et al., 1978; Krishnan and Humphrey, 1986) whereas replenishment of an irradiated host with bone marrow stem cells results in adequate protection (Tripathy and Mackaness, 1969; McGregor and Koster, 1973; Takeya et al., 1977). However, if the monocytes are provided with factors such that they mature into fully active macrophages, the ability to kill Listeria is again decreased (Czuprynski et al., 1983).

In humans, the recruited neutrophils exhibit the greatest level of bactericidal activity of the three cell

types discussed here (Steigbigel et al., 1974; Peterson et al., 1977). The listericidal mechanism has still not yet been fully elucidated. Studies of the role of neutrophils in animal models of Listeria resistance are limited (Tatsukawa et al., 1979), but it is known that these cells are responsive to lymphokines (Rocklin and Rosenthal, 1977) and infiltrate the site of infection during the early stages of listeriosis (North, 1970; Mandel and Cheers, 1980). Unlike mouse PEM, neutrophils contain MPO and thus are able to utilize chlorinium and iodinium ions for killing in addition to direct oxidative mechanisms.

Thus, the process by which a host resists a challenge of viable Listeria is composed of a complex series of sequential events. This includes (1) the initial capture and killing of Listeria by a highly bactericidal subpopulation of resident macrophages, (2) antigenic processing and stimulation of naive T-cells mediated through the macrophage Ia molecules of the major histocompatibility complex, (3) T-cell clonal expansion and release of lymphokines such as colony-stimulating factor and gamma-interferon, (4) the maturation and recruitment of bone marrow monocytes and neutrophils as well as the activation of the resident macrophages, and (5) leukocyte capture and killing of proliferating Listeria via activated oxygen metabolites or by non-oxidative mechanisms, possibly cationic proteins (Patterson-Delafield et al., 1980; Lehrer et al., 1981).

CHAPTER III
EFFECTS OF 6-WEEK VANADATE EXPOSURE ON MOUSE WHOLE BODY CLEARANCE AND THE MACROPHAGE UPTAKE AND INTRACELLULAR KILLING OF *LISTERIA MONOCYTOGENES*

Introduction

Many chemicals, especially environmental pollutants, known carcinogens/teratogens, and metals have been evaluated *in vitro* and *in vivo* for their immunomodulating effects. Often an agent might specifically target the humoral immune system or the cell-mediated immune system only. Fewer compounds display a broad effect upon both systems, and often the immunotoxic effect is the result of targeting of one cellular component crucial to the resulting function of both systems (i.e. loss of T-cell differentiation resulting in non-maturation of B-lymphocytes to immunoglobulin-secreting plasma cells). Effectors of macrophage function may also bring about this broad spectrum of immune dysfunction; this cell is the primary site for antigenic processing and the conferring of specificity in either type of immune response.

Several test systems exist for assaying the immunomodulating potential of suspect chemicals. Commonly, antibody formation and resistance to bacterial/viral lethality are measured *in vivo*, while lymphoproliferative responses to mitogens and particle clearance are standard *in vitro* assays. In several cases, a particular immune response (i.e. cell-mediated) can be assayed *in vivo* using agents

which are known to be specific inducers. Listeria monocytogenes is useful in this respect in that: (1) host resistance is specifically a cell-mediated event, (2) an immune response occurs early (< 4 days) such that immunomodulation is quickly revealed, and (3) bacterial enumeration provides an accurate quantitative assessment of the magnitude of immunomodulation (Dean et al., 1982b).

Loss of resistance to Listeria has been shown with known immunosuppressive agents such as diethylstilbestrol, flumethazone, cyclophosphamide, and delta-9-tetrahydrocannabinol (Bradley and Morahan, 1982; Dean et al., 1982b). Other studies with agents that resulted in decreased host resistance, such as carageenan and silica (Mitsuyama et al., 1978; Zimmerman et al., 1986) have indicated that immunotoxicity occurred at the level of the macrophage. Tam and Hinsdill (1984) tested a wide variety of immunomodulating chemicals and noted that decreased microbicidal activity was the result of defects in macrophage phagocytosis. Other studies have since shown that certain metals, such as cadmium and lead, exert their macrophage-modifying effects by disturbing the process of phagocytosis directly or by inhibiting energy-producing pathways required for phagocytosis (Castranova et al., 1980; Hilbertz et al., 1986).

In 1974, Waters et al. demonstrated that the toxic effects of vanadium salts on rabbit alveolar macrophages were dependent upon solubility parameters. Valence did not correlate with cytotoxicity although the pentavalent

vanadium ion is the most toxic form of the metal in the intact animal. Castranova et al. (1984) reported that trivalent vanadium was more cytotoxic than other valence states, but this was likely due to its strong reducing potential. Although macrophage phagocytic activity was decreased following in vitro pentavalent vanadium exposure, the ability to attach to surfaces and viability were unaffected (Fisher et al., 1978). Reduced phagocytic activity was thought to be the result of vanadium-induced morphologic alterations of the macrophage membrane (Waters and Gardner, 1975) so that normal pseudopodia extension and fusion around antigen particles were diminished.

Although reduced phagocytic activity will contribute to a decreased resistance to Listeria, the effects of test agents on intracellular killing functions have received less scrutiny. Not only have studies with silica (Zimmerman et al., 1986), platinum (Sodhi and Gupta, 1986) and strontium (Ackermann and Morahan, 1988) demonstrated the effects of these agents on the uptake of microorganisms, but also on the resulting oxygen metabolite activation and/or detoxification pathways. In studies with vanadium, Wei and Misra (1982) demonstrated similar effects by monitoring chemiluminescent responses in isolated macrophages. Further studies with mice exposed to vanadium showed that the macrophages displayed decreased phagocytic functions more than 3 days after the last dose of metal (Cohen et al., 1986).

In the latter study, mice pretreated with vanadium also displayed dramatically reduced resistance to challenge with Listeria. The underlying mechanisms by which vanadium, as well as other metals, induced decreased resistance to this pathogen have not been fully explored. The possible intracellular effects upon energy production for phagocytic activity and upon oxygen metabolite activation for killing could be combined with previously described vanadium-induced structural defects in order to develop a mechanism for the decreased resistance. By monitoring bacterial clearance patterns in the infected host, as well as the phagocytic and intracellular killing activities of isolated macrophages against Listeria, a more precise target for vanadium immunomodulation of the cell-mediated response can be identified.

Materials and Methods

Dosing regimen

Female $B_6C_3F_1$ mice (Harlan Sprague Dawley Co., Indianapolis, IN) weighing 18-20 grams (6-8 weeks old) were placed three to a cage and allowed to acclimate for 1 week. Mice were kept in an animal room maintained at 70-75°F that provided 12 hr of light daily. Mice were fed Ralston Purina Lab Chow 5001 (Ralston Purina Co., St. Louis, MO) and water ad libitum. Mouse body weights were recorded weekly to determine appropriate doses of vanadium or control solutions.

Ammonium metavanadate (NH_4VO_3) was dissolved in 0.1 M phosphate buffer (pH 7.2) to yield solutions of 1.0 or 0.25 mg V/ml buffer. "V" is equivalent to the metal atom and not the compound or the vanadate ion. The 1.0 mg V/ml solution was prepared by dissolving 2.30 g NH_4VO_3 (J.T. Baker Co., Philipsburg, NJ) per liter of buffer and warming the solution over a low-heat source. The 0.25 mg V/ml solution was prepared using 0.56 g of the compound. Solutions were filtered and stored in sterile 15 ml vacutainer tubes (Vacutainer Systems, Rutherford, NJ). All solutions were refrigerated and brought to room temperature before injection. These concentrations allowed for delivery of 2.5 and 10.0 mg V/kg body weight in volumes less than 1 ml. These doses were equal to 1/8 and 1/2 of the IP LD_{50} for this mouse strain.

Ammonium chloride solution was prepared as a control to yield the equivalent amount of ammonium ion given to the mice receiving the highest vanadium treatment (10V), 1.05 mg NH_4Cl/ml buffer. The vehicle phosphate buffer served as another control solution.

After the first week, the mice were separated into 4 groups: 10.0 mg V/kg (10V), 2.5 mg V/kg (2.5V), phosphate buffer or ammonium chloride. After weighing, the mice received the respective solutions by intraperitoneal (IP) injection. This treatment regimen was repeated every 3 days for a period of 6 weeks; dosing every 3 days allowed for the

maximal excretion of the previous dose prior to injection (Hopkins and Tilton, 1966; Sabbioni and Marafante, 1978).

Peritoneal macrophage harvesting

Three days following the final injection, the mice were sacrificed by cervical dislocation and the peritoneal macrophages (PEM) were harvested. Following sacrifice, the abdomen was swabbed with ethanol and the fur removed. Care was taken not to puncture the peritoneal cavity; if this occurred, the mouse was discarded. The outside of the cavity was rinsed with ethanol to remove any hairs or debris. Three ml of ice-cold sterile Hanks' balanced salt solution (HBSS, pH 7.5, from freshly prepared stock (Rovozzo and Burke, 1973)) was injected into the cavity by inserting a syringe with a 20-gauge needle just below the xyphoid, along the mid-anterior line. The cavity was distended and the solution gently mixed by prodding of the cavity by hand. An 18-gauge needle was inserted into the extreme right flank of the cavity, just above the spleen and the syringe was pulled slightly away from the body so as to form a pocket of fluid. The peritoneal fluid was withdrawn and transferred to a sterile 15 ml centrifuge tube held on ice. This process was repeated 5 times to yield 13-15 ml of peritoneal fluid/mouse. The entire process was repeated with the other two mice in the treatment group. On each of the 4 days of experiments, 3 mice/treatment were sacrificed until a total of 12 mice/treatment were analyzed.

The PEM were pelleted by centrifugation at 1200 x g for 10 min at 4°C. The supernatants were decanted and the cells were resuspended in fresh cold HBSS (3 ml). The cell suspensions from three mice were combined to yield one cell population per treatment group per experiment. The pooled suspension was pelleted and resuspended in 3 ml of the buffer used in the particular experiment. A 0.1 ml aliquot was removed and mixed with 0.9 ml of 0.4% trypan blue solution. The population viability and concentration was determined on a hemacytometer (American Optical, Buffalo, NY). A second 0.1 ml aliquot was removed and examined to verify the first results. Using non-specific esterase staining (Stuart et al., 1975), preliminary studies confirmed that the yields were 85%-90% macrophages and/or monocytes.

Clearance of Listeria monocytogenes from the Peritoneal Cavity, Liver and Spleen

Two days after the final dose of vanadium or control solutions, the mice were injected IP with a sublethal dose of viable Listeria monocytogenes. The dose, 2.4×10^4 bacteria per mouse, was selected based upon the previously determined decrease in the LD_{20} for Listeria when this strain of mouse received the 2.5V dosage for only 3 weeks (3.3×10^3 bacteria per mouse) as well as on the basis of preliminary studies to determine the level of organism which can be accurately measured in the present assay system.

The Listeria cultures were prepared from colonies maintained on trypticase soy agar slants (TSA, Difco Inc., Detroit, MI). The colonies were inoculated into trypticase soy broth (TSB, Difco Inc.) and grown overnight at 37°C. The concentration of the stock was calculated based upon the absorbance of a 1:1 dilution at 540 nm using an absorptivity of 7.44×10^{-10} bacteria$^{-1} \cdot$ cm$^{-1} \cdot$ ml. The working solution, 1×10^{5} Listeria/ml, was prepared by dilution of the stock with TSB. This solution was plated onto TSA supplemented with 0.6% yeast extract (w/v) in order to confirm the initial dosage given each animal.

Each mouse was injected with the bacteria in a 0.2 ml volume of TSB. The injection site was massaged to insure dispersion of the organism throughout the peritoneal cavity. After 10 min, mice were sacrificed by cervical dislocation and the abdominal area was immediately swabbed with alcohol. The peritoneal cavity was exposed as described earlier and 6 ml of sterile HBSS containing 0.1% gelatin (HBSS:gel, pH 7.5) was injected. After gentle hand-prodding to allow for adequate mixing in the cavity, the solution was withdrawn and transferred to a sterile centrifuge tube held on ice. The peritoneal solution was maintained on ice until plating so as to prevent the phagocytosis of non-ingested organisms. The solution was plated directly or serially diluted with phosphate buffered saline (PBS, pH 7.4) before plating in quadruplicate onto the modified TSA plates. The plates were then inverted and incubated for 24 hr to allow for colony

counting. Each treatment group consisted of four animals per time period. At 10 min, 4, 8, 12, 24, 48 and 72 hr post-infection, the process as described above was performed.

After removal of the peritoneal fluid, the liver and spleen of each mouse was aseptically removed. Each organ was rinsed thoroughly in 2 washes of sterile PBS before blot-drying on sterile gauze held in glass petri dishes. The semi-dry liver or spleen was then weighed and transferred to a test tube holding 9 or 5 ml of PBS, respectively, for homogenization. The sample was homogenized with the Polytron (Brinkmann Instruments, Westbury, NY) and the rotary blade was washed with sterile distilled water between each sample. The homogenate of each organ was then plated directly or serially diluted with PBS before plating onto TSA. The organ weight and the homogenate volume were recorded and used for later calculation of the total bacterial number present. On the basis of the number of Listeria present, the pattern of trapping and clearance of the organism by the mouse could be monitored as a function of the length of infection.

Listeria phagocytosis by peritoneal macrophages

The modified procedure of Leijh et al. (1984) was used to monitor the phagocytosis of Listeria by the PEM. Two days after the final injection of vanadium or control solutions, the mice were sacrificed and the PEM collected. The pooled cells were washed twice and counted with a

hemacytometer before being suspended at a final concentration of 5×10^6 PEM/ml in HBSS:gel solution. A working solution of 5×10^6 Listeria/ml TSB was prepared as above from an overnight stock culture and 2 ml of this solution was placed in a sterile silicon-coated vacutainer tube. A 0.4 ml aliquot of intact whole mouse serum (pooled mouse serum, Cappel Labs, West Chester, PA) was added and the solution mixed. Two ml of cell suspension was added and the sample was again mixed before placing in a 37°C water bath with shaking at <10 rev/min.

Before the sample was placed in the bath, a 0.5 ml aliquot was removed and added to 1.5 ml of ice-cold HBSS:gel held on ice. The bacteria:PEM mixture was then centrifuged at 100 x g for 4 min and a 0.5 ml aliquot of the supernatant was carefully removed. Under these conditions, the non-ingested bacteria remain in the supernatant (Leijh et al., 1981). The supernatant sample was then serially diluted and plated in quadruplicate onto the TSA plates; this sample was designated as t_0. The process was repeated again at 30, 60, 90, and 120 min post-mixing. For each treatment group, 2 sets of PEM:bacteria mixtures were prepared and analyzed. In order to account for bacterial proliferation by non-ingested Listeria, a control without PEM but with HBSS:gel was prepared and analyzed as above.

To obtain a more definite measurement of the phagocytosis by the harvested PEM, aliquots of the bacteria:PEM solution were removed at 60 and 120 min

post-mixing and washed several times with fresh HBSS:gel and repeated centrifugation. The final pellet was reconstituted in a small volume (0.5 ml) of the HBSS:gel and aliquots were placed on clean glass microscope slides precoated with 0.5% bovine serum albumin. Smears were then prepared and the slides allowed to air dry. The slides were then fixed with methanol for 1 min and air-dried for later staining.

The standard procedure for analysis of phagocytosis is via morphological assessment (Ratzan et al., 1972; Leijh et al., 1984) using Wright-Giemsa staining of the phagocytes. To take advantage of the gram-positive nature of Listeria so that differential staining of the cells and organism could aid in microscopic analyses, the smears were stained first with crystal violet solution for 1 min. After washing with distilled water, the slides were air-dried and PEM were counterstained with aqueous methyl green (1%, w/v) for 4 min before a final water rinse and air-drying. The slides were then sealed with a coverslip and examined with a microscope using high magnification (1500 X). For each treatment and time period, a minimum of 5 slides were prepared. Each slide was examined for free bacteria, PEM, and PEM containing ingested Listeria. Differentiation between ingested bacteria and those adhering to the cell surface was performed by varying the field depth. At least 150 PEM per slide were examined and the number of bacteria per PEM was measured in order to arrive at a phagocytic index.

Intracellular killing of Listeria by peritoneal macrophages

The modified method of Leijh et al. (1984) employing macrophage-Listeria suspensions was used for measuring intracellular killing in the freshly harvested cells. Initial studies were performed using PEM attached to glass coverslips according to the method of Harrington-Fowler et al. (1981) and Cole (1975); levels of bacteria taken up by the attached cells were small (less than 1% as predicted by Czuprynski et al., 1983) and inadequate for estimating differences in killing as a function of host pretreatment.

Macrophages were harvested as described earlier and the final concentration adjusted to 2×10^6 PEM/ml of complete medium without antibiotic-antimycotic solution [CM(-)]. A freshly grown sample of Listeria was diluted with CM(-) and opsonized by incubating with whole mouse serum (Cappel Labs, 10% by total volume) at 37°C for 30 min. The bacterial suspension was adjusted to a final concentration of 5×10^6 Listeria/ml and an aliquot removed for plating on modified trypticase soy agar plates in order to confirm the starting bacterial levels. To each of 3 silicon-coated tubes prepared for each mouse treatment, 2 ml of the bacterial suspension was added followed by the addition of 2 ml of the cellular suspension. Each tube was then stoppered with a foam-rubber plug and placed in a waterbath maintained at 37°C and rotated at 5 rpm. After allowing 20 min for phagocytosis of the opsonized bacteria, the contents of the

tube were transferred to a sterile plastic centrifuge tube. Non-ingested bacteria were removed by centrifugations at 150 x g (3 times). After the first centrifugation, 3.5 ml of the supernatant was carefully removed and transferred to a separate test tube for studies to confirm ingestion. Two ml of fresh CM(-) was added, the pellet was resuspended, and centrifugation for 5 min at 150 x g was repeated. Two ml of supernatant was removed, the process was repeated and the two wash volumes were combined with the original 3.5 ml. The total volume was then recorded, and the sample was serially diluted and plated on TSA. After the third wash, the PEM-bacteria pellet was resuspended in a total volume of 4 ml CM(-) and the contents were transferred back to a sterile foam-stoppered silicon-coated tube.

After the removal of the non-phagocytized bacteria, the PEM-*Listeria* suspension was placed back into the 37°C waterbath and shaken at 5 rpm. A 0.5 ml aliquot of the suspension was then removed and added to 0.5 ml of ice-cold CM(-) in a centrifuge tube held on ice in order to halt intracellular killing. The sample was vortexed and centrifuged for 5 min at 150 x g to displace any loosely adhering bacteria from the cell membrane. After removing and discarding 0.9 ml of supernatant, and washing again with 1 ml fresh medium, 1 ml of ice-cold aqueous bovine serum albumin (0.01%, w/v) was added. The pellet was resuspended and the content was transferred to a glass test tube. The PEM suspension was then frozen and rapidly thawed 3 times

using a dry ice:acetone system so that surviving intracellular Listeria could be serially diluted and plated in quadruplicate on TSA. The freeze-thaw process had little effect on bacterial viability as shown in a preliminary study. This sample represented the t_0 value, and sampling was repeated at t = 2, 4, 8 and 12 hr post-phagocytosis.

To prevent the possible reingestion of Listeria released from dead PEM, the suspensions were washed every hour to remove free bacteria. The content was transferred to a sterile centrifuge tube and pelleted (5 min, 150 x g). After removing the supernatant, fresh CM(-) was added so that the volume was the same as before washing (not equivalent to the original 4 ml due to aliquot removal for samplings).

A PEM-free control was prepared to measure uncontrolled bacterial growth with sampling at the above time points. An additional bacteria-free control was prepared in order to measure PEM viability over the entire study period. A 0.1 ml aliquot of this cell suspension was removed at 1, 3, 5, 7 and 10 hr post-mixing and combined with 0.1 ml of trypan blue. The total number of live and dead PEM was measured in 4 quadrants of a hemacytometer in order to determine overall PEM survival. To measure any loss in PEM levels due to bacterial lysis, at t = 0, 4, 8 and 12 hr post-phagocytosis, the PEM-Listeria suspension was transferred to a centrifuge tube and pelleted (5 min, 150 x g). The supernatant was removed and the volume of the suspension

adjusted to 1 ml. After resuspension, 0.05 ml was removed and smears were prepared using slides previously coated with 0.5% BSA (see above). The staining procedures used in the phagocytosis study were used and the total number of PEM in a fixed zone was counted.

Statistical analysis

The statistical significance of the observed phagocytic rates as well as the intracellular killing at the select time periods was determined using an analysis of variance combined with a Duncan's analysis of the means test. The data obtained for each separate experiment was analyzed independently and then the averages pooled to determine trends over the entire experimental period.

Results

The study of the clearance of viable Listeria from the peritoneal cavity of treated mice indicates that animals exposed to vanadium for 6 weeks showed a dose-dependent significant ($P < 0.01$) increase in organism levels in the liver and spleen, and also displayed higher levels at time points much earlier than in the control mice (Figures 4 and 5). As indicated in Table 1, the IP dosing of 2.4×10^4 viable organisms did not allow for detectable levels of Listeria in the control livers until 24 hr post-injection. However, mice pretreated with the 10V regimen had detectable hepatic levels as early as 8 hr post-infection, while 2.5V mice had detectable levels at 12 hr. A similar pattern of

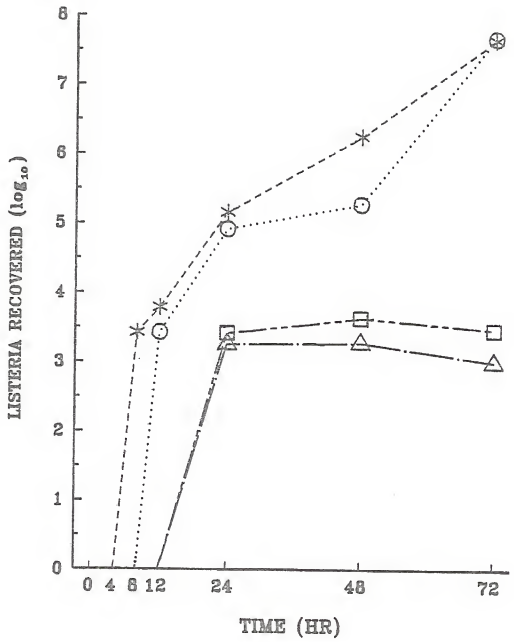


Figure 4. Listeria recovered in liver as a function of time. Each point is the mean logarithmic number of recovered Listeria from four mice per treatment group/time period following initial infection with 24000 organisms/mouse. Treatment regimens (6-week) are as follows: 10 mg V/kg (*), 2.5 mg V/kg (O), ammonium chloride (Δ), and phosphate buffer (□).

Table 1. The effects of 6-week pretreatments on body weight (BW), liver weight (LW) and index (LI), and on levels of viable *Listeria* after host infection.

HR	BW[a]	LW[a]	LI	$\log_{10}$ LM	BW[a]	LW[a]	LI	$\log_{10}$ LM
		10V					N^+	
0	24.6±2.5	1.5±0.3	6.1±0.7	---	23.9±2.0	1.4±0.1	6.0±0.1	---
4	23.2±1.4	1.2±0.1	5.3±0.6	---	22.6±1.8	1.2±0.1	5.3±0.4	---
8	23.3±1.1	1.3±0.1	5.4±0.4	3.4 ± 0.3^{v}	22.6±0.7	1.2±0.1	5.1±0.2	---
12	24.0±1.9	1.4±0.2	5.9 ± 0.4^{e}	3.8 ± 0.2^{v}	23.0±2.6	1.2±0.2	5.3±0.2	---
24	22.8±0.3	1.5±0.1	6.6 ± 0.3^{e}	5.2 ± 0.2^{x}	22.8±0.4	1.4±0.0	6.0±0.1	3.3±0.4
48	19.5 ± 1.4^{ef}	1.4±0.1	7.1 ± 0.1^{ef}	6.3 ± 0.2^{y}	24.5±0.9	1.5±0.1	5.9±0.3	3.3±0.1
72	18.2 ± 0.9^{ef}	1.3±0.1	7.2 ± 0.4^{ef}	7.7 ± 0.4^{z}	23.1±1.6	1.4±0.1	6.2±0.3	3.0±0.0
		2.5V					P	
0	22.7±0.7	1.3±0.1	5.7±0.3	---	22.4±0.9	1.3±0.1	5.6±0.1	---
4	23.2±2.2	1.3±0.1	5.6±0.3	---	23.1±0.5	1.2±0.1	5.1±0.4	---
8	23.6±1.6	1.3±0.1	5.6±0.3	---	22.6±0.6	1.2±0.0	5.4±0.2	---
12	23.2±1.0	1.2±0.1	5.3±0.2	3.4 ± 0.5^{x}	23.2±2.7	1.2±0.2	5.4±0.3	---
24	23.4±1.6	1.4±0.2	6.2±0.4	4.9 ± 0.5^{y}	22.8±1.7	1.3±0.2	5.7±0.4	3.4±0.4
48	21.9±1.3	1.4±0.1	6.5 ± 0.4^{f}	5.3 ± 0.4^{y}	23.8±1.6	1.6±0.1	6.6 ± 0.5^{f}	3.6±0.2
72	19.1 ± 1.1^{ef}	1.2±0.1	6.4 ± 0.8^{f}	7.7 ± 0.3^{z}	22.6±1.9	1.4±0.1	6.3±0.3f	3.5±0.3

[a]Value in grams (± SD)

[e]Values are significantly different from controls or [f]from 0-Hour value at $P < 0.05$.

[v,x,y,z]Values are significantly different within same treatment at $P < 0.01$.

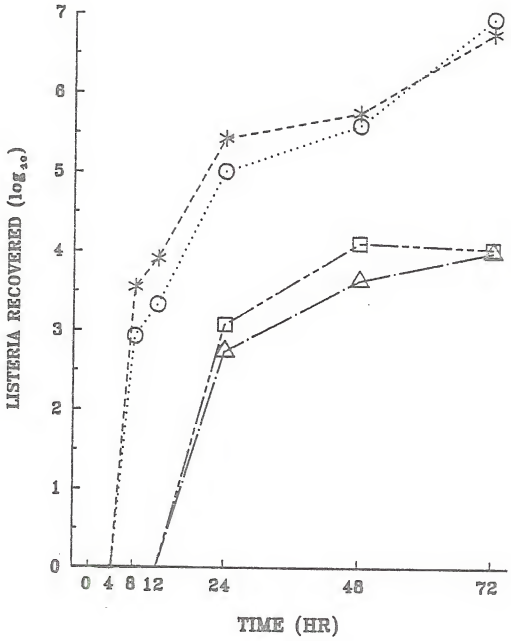


Figure 5. Listeria recovered in spleen as a function of time. Details for figure were outlined in Figure 4.

Table 2. The effects of 6-week pretreatments on body weight (BW), spleen weight (SW) and index (SI), and on levels of viable *Listeria* after host infection.

HR	BW[a]	SW[a]	SI	$\log_{10}$ LM	BW[a]	SW[a]	SI	$\log_{10}$ LM
		10V				N+		
0	24.6±2.5	0.09±0.02	0.38±0.08	---	23.9±2.0	0.08±0.02	0.34±0.08	---
4	23.2±1.4	0.08±0.01	0.37±0.02	---	22.6±1.8	0.09±0.02	0.38±0.07	---
8	23.3±1.1	0.09±0.02	0.41±0.12	3.6 ± 0.2^{x}	22.6±0.7	0.08±0.01	0.35±0.04	---
12	24.0±1.9	0.10±0.04	0.43±0.16	3.9 ± 0.3^{x}	23.0±2.6	0.09±0.03	0.41±0.07	---
24	22.8±0.3	0.11±0.01	0.47±0.03	5.4 ± 0.4^{y}	22.8±0.4	0.09±0.03	0.47±0.04	2.7 ± 0.4^{y}
48	19.5±1.4	0.10±0.02	0.51±0.08	5.8 ± 0.3^{y}	24.5±0.9	0.12±0.01	0.51±0.05	3.6 ± 0.4^{z}
72	18.2±0.9	0.09±0.01	0.52±0.05	6.8 ± 0.3^{z}	23.1±1.6	0.14±0.02	0.63±0.07	4.0 ± 0.2^{z}
		2.5V				P		
0	22.7±0.7	0.08±0.01	0.38±0.03	---	22.4±0.9	0.08±0.01	0.37±0.04	---
4	23.2±2.2	0.12±0.04	0.52±0.19	---	23.1±0.5	0.08±0.02	0.36±0.09	---
8	23.6±1.6	0.10±0.02	0.42±0.07	2.9 ± 0.2^{x}	22.6±0.6	0.10±0.02	0.46±0.10	---
12	23.1±1.0	0.08±0.02	0.36±0.10	3.3 ± 0.5^{x}	22.6±1.9	0.15±0.02	0.64±0.06	4.2±0.4z
24	23.4±1.6	0.09±0.01	0.41±0.02	5.0 ± 0.8^{y}	22.8±1.7	0.09±0.01	0.39±0.01	3.1 ± 0.1^{y}
48	21.9±1.3	0.12±0.02	0.57±0.08	5.6 ± 0.3^{y}	23.8±1.6	0.12±0.01	0.52±0.02	4.1 ± 0.4^{z}
72	19.1±1.1	0.09±0.01	0.47±0.06	6.9 ± 0.3^{z}	22.6±1.9	0.15±0.02	0.64±0.06	4.2±0.4z

[a]Value in grams (± SD)

v,x,y,zValues are significantly different within same treatment at $P < 0.01$.

splenic Listeria populations was apparent, with both vanadium-treated groups having detectable levels by 8 hr post-infection while controls did not have detectable organisms until the 24 hr time point (Table 2). In both studies, the organ levels of Listeria in vanadate-treated mice were significantly ($P < 0.01$) increased with an increasing length of infection. In control mice, there was a significant rise in splenic burdens from 24 to 48 hr post-infection, but none thereafter.

Following injection of the Listeria, the hepatic and splenic indices of all the treatment groups were increased. The increase in hepatic indices by 48 hr was significant ($P < 0.05$) in all but the NH_4Cl group. After 12 hr of infection, the 10V mice had significantly ($P < 0.05$) higher hepatic indices as compared with the controls. At the later time points assayed, body weights of the vanadate-treated mice were significantly lower than the controls ($P < 0.05$).

The clearance of Listeria from the peritoneal cavity was dramatically affected by vanadium treatment of the host. By 4 hr post-infection, the control mice had completely eliminated the bacterial challenge (Table 3) while detectable levels in vanadium-treated mice reached a nadir. The vanadium-treated mice displayed time-dependent significant ($P < 0.01$) increases in the assayable levels of the organism over the remaining period of the assay (Figure 6). By 24 hr post-infection, the 10V mice had levels significantly higher than the 2.5V mice ($P < 0.01$); by 48 hr, 10V levels of

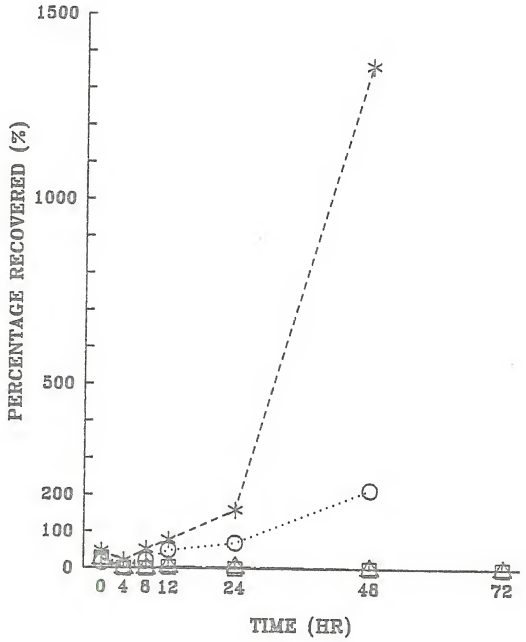


Figure 6. Percentage recovery of initial Listeria dosage as a function of time. Each point was the mean total number of viable Listeria recovered in a 6 ml HBSS wash of the peritoneal cavity at the times indicated. Values are expressed as the percentage of the initial infecting dose (equivalent to 4000 Listeria/6 ml HBSS injected). Mouse pretreatment regimens were described in Figure 4.

Table 3. The effects of pretreatment on clearance of *Listeria* from the peritoneal cavity.

HR	[a]LM/ml	FLUID VOLUME	% LM INITIAL[b]	[a]LM/ml	FLUID VOLUME	% LM INITIAL[b]
	10V			N+		
0	1.9±0.8	5.7±0.2	47.0±18.8	0.9±0.1	5.6±0.2	23.8±18.2
4	0.8±0.1	5.4±0.3	20.9±3.5	0	5.6±0.1	0
8	3.6±0.3	5.3±0.3	52.2±10.2	0	5.4±0.3	0
12	3.1±0.9	5.3±0.3	78.2±22.7	0	5.3±0.2	0
24	6.4±1.3	5.4±0.6	159.8±54.4[x,*]	0	5.4±0.1	0
48	54.5±6.2	5.3±0.2	1362.0±155.[y,*]	0	5.7±0.1	0
72	702.0±33.6	5.3±0.4	24562.0±1181[z]	0	5.5±0.2	0
	2.5V			P		
0	0.6±0.1	5.6±0.1	16.0±3.3	1.1±0.4	5.9±0.1	27.8±9.1
4	0.2±0.1	5.4±0.3	4.3±2.9	0	5.4±0.2	0
8	0.9±0.3	5.4±0.1	22.5±15.7	0	5.1±0.2	0
12	2.0±0.1	5.1±0.4	49.9±32.4	0	5.2±0.2	0
24	2.8±0.3	5.4±0.2	70.1±7.9[x]	0	5.6±0.1	0
48	8.6±1.2	5.6±0.1	214.8±31.4[y]	0	5.4±0.2	0
72	630.0±25.4	5.7±0.2	22105.0±8922.0[z]	0	5.1±0.2	0

[a]Viable *Listeria* (x 10^3) recovered per ml of peritoneal fluid.

[b]Initial levels of *Listeria* equal to 4000 organisms/ml HBSS injected.

[x,y,z]Value significantly different within same treatment or [*]from all others at $P < 0.01$.

bacteria were 1300% higher than the initial dose while 2.5V levels were only 200% higher. However, by 72 hr post-infection, both sets of vanadium-treated mice had peritoneal levels >20,000% of the initial dose (Table 3; these data points not shown in Figure 6). The patterns of Listeria growth in the three sites examined were similar to patterns (excluding the ammonium chloride group) for Listeria mortality observed earlier (Figure 7) (Cohen et al., 1986).

The study of the phagocytosis of the live bacteria in a suspension of freshly harvested PEM made apparent the difficulties in trying to analyze a single step in a very complex series of events. As the length of coincubation of Listeria with PEM increased, the total number of organisms was relatively constant for the first 60 min, but then increased until the 120 min timepoint (Figure 8). There was no discernable pattern of growth that could be related to the pretreatment the hosts had received.

When individual preparations of the suspensions were analyzed microscopically, the phagocytic indices of the freshly harvested PEM were more readily measured. After 60 min of coincubation, the PEM from the control mice were approximately 23% positive (Figure 9), with positive indicating that the PEM had one or more Listeria within the cell (and not simply adhering to the membrane). The vanadium treated mice had a dose-dependent significantly lower ($P < 0.05$) percentage of positive cells with the 2.5V and 10V mice having 19.6% and 17.0% values. After 120 min of

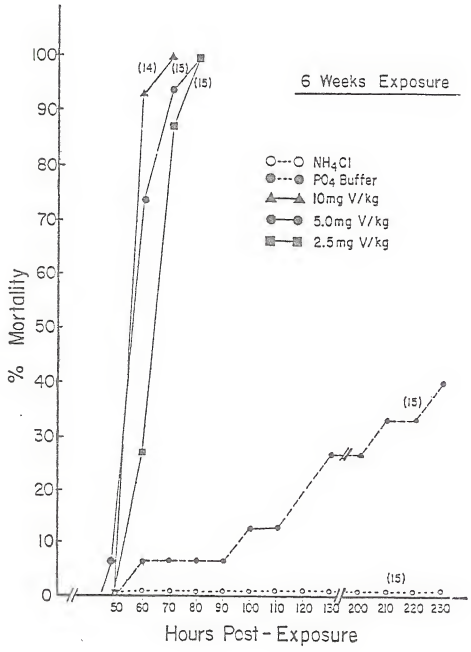


Figure 7. Percent mortality versus time of death (cumulative) after Listeria exposure. Mice received the indicated doses every 3 days for 6 weeks prior to infection. Values represent the percentage of the total population in each treatment group that succumbed after initial infection with 8.4×10^5 Listeria. Number in parentheses are total number of mice in each test group. (Reproduced from Cohen et al., 1986).

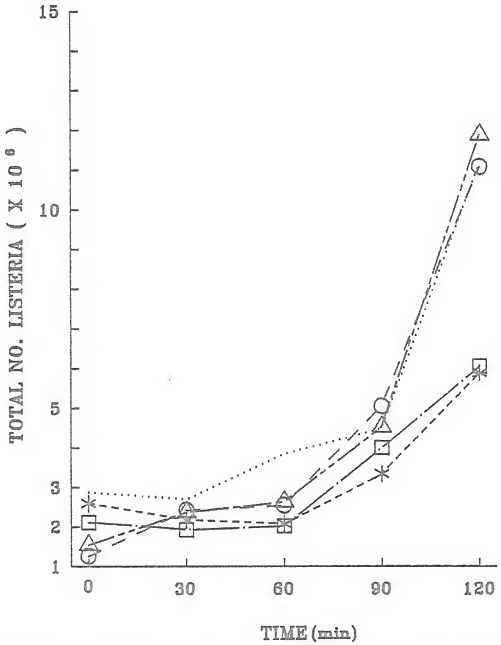


Figure 8. Listeria levels as a function of the length of coincubation with mouse peritoneal macrophages. Each point represents the average number (x 10^6) of non-ingested Listeria recovered from two separate bacteria:macrophage systems (1:1 degree of infectivity) at the designated time points. Listeria were isolated from incubates containing macrophages recovered from pretreated mice: 10 mg V/kg (Δ), 2.5 mg V/kg (□), NH_4Cl (*), phosphate buffer (O), or in a macrophage-free system containing serum (·····).

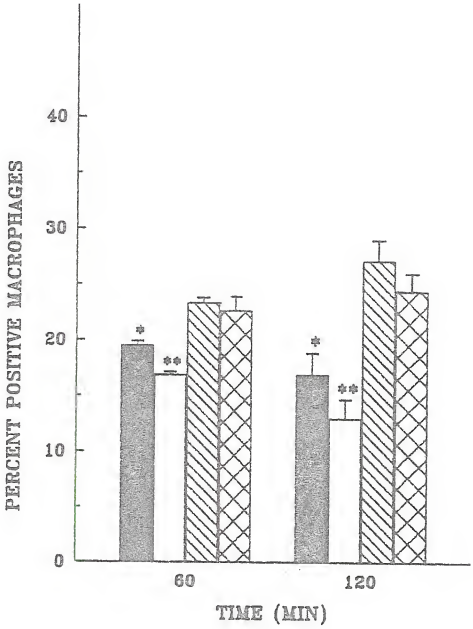


Figure 9. Percentage of macrophages containing one or more Listeria. Each bar is the mean (± SD) number of positive macrophages observed from examination of 5 slides/treatment (150 cells/slide) after the designated length of cell culture infection. Values for 10V (▭) and 2.5V (▬) mouse cells significantly different from the buffer (▨) and NH_4Cl (▨) controls at $P < .05$ (*) or from each other (**) are noted.

coincubation, the percentage of positive PEM increased for the controls (to levels of 24.5%-27.3%), while levels for the vanadium treated mice showed dose-dependent decreases (to 17% and 13%, respectively). In no case were the differences in the percentages from 60 to 120 min within each treatment significant.

During the process of evaluating the cells for positive status, the PEM were also scored for the number of organisms ingested. In both time periods analyzed, there were no apparent differences among the different pretreatments in the population distributions of the bacteria ingested, although the absolute number of positive cells differed (Figures 10 and 11).

The freshly harvested PEM were also assayed for intracellular killing capabilities. Preliminary studies using PEM attached to glass coverslips maintained in Leighton tubes resulted in very low uptake of the inoculi. Switching to a system where the PEM and the bacteria were in suspension allowed for ingestion levels on the order observed in the phagocytosis study (approximately 25% for the controls). Initially, the use of an antibiotic-antimycotic solution was contemplated, but a preliminary study indicated no detectable bacteria within 2 hr of coincubation, not even in the cell lysates. The presence of traces of antibiotic on the modified TSA plates was adequate to block the growth of an expected high number of organisms. A preliminary time-course study of the effect of the antibiotic on freely

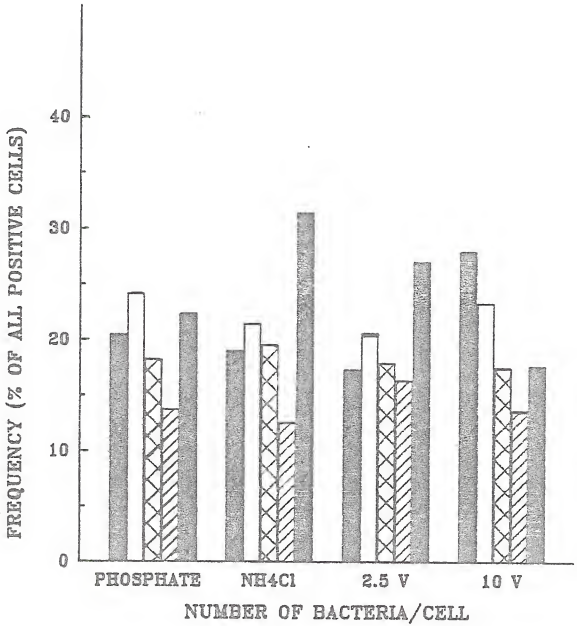


Figure 10. Population distribution of Listeria in macrophages after 60 min. Frequency values for macrophages containing 1, 2 ,3, 4 or ≥5 Listeria (bars from left to right within each cluster) are indicated. Each cluster represents the average total frequencies obtained from 10 slides per treatment group.

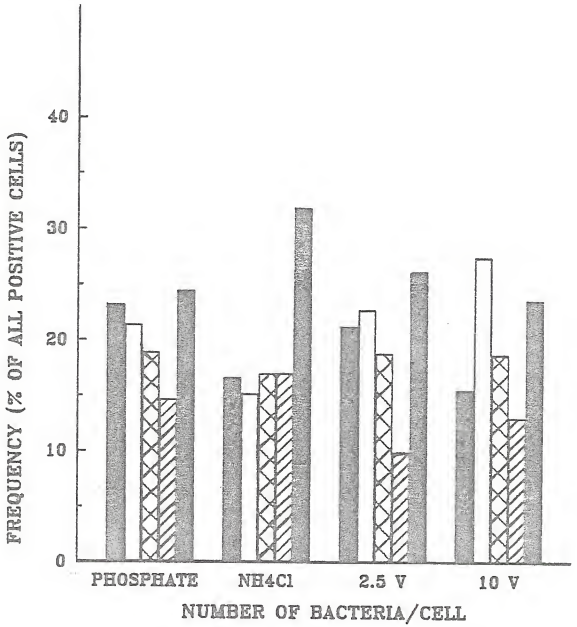


Figure 11. Population distribution of Listeria in macrophages after 120 min. Details in Figure 10.

growing Listeria indicated that after 5 min of coincubation there was a 20% decline in the numbers of viable organisms. This decrease continued with time and by the end of 20 min coincubation, the decrease was >99% (data not shown).

Therefore, the resulting assay system was dependent upon the washing away of non-ingested Listeria to prevent possible reingestion following cell death. The suspensions were washed hourly and the PEM were resuspended in fresh antibiotic-free medium each time. The washing procedure was also not 100% effective; preliminary pelleting experiments with pure Listeria suspensions indicated that three successive centrifugations at 150 x g (for 5 min each) resulted in the removal of 85% of the total bacteria present. Pelleting at higher speeds (1200 x g) resulted in 78% removal (data not shown). Centrifugation of only PEM for the same lengths of time and at the same speeds consistently resulted in 100% recovery of the phagocytes.

The PEM were assayed for intracellular killing capacities over a 12 hr period. Three of the pretreatments resulted in similar time-related patterns of total viable intracellular organisms (Figure 12). Only the PEM from ammonium chloride-treated mice did not display an increase in intracellular levels in the first 2 hr post-phagocytosis, but these cells did show the slow increase and subsequent leveling in Listeria numbers after 4 hr. During the first 2 hr post-infection (t_0-t_2), the levels of viable

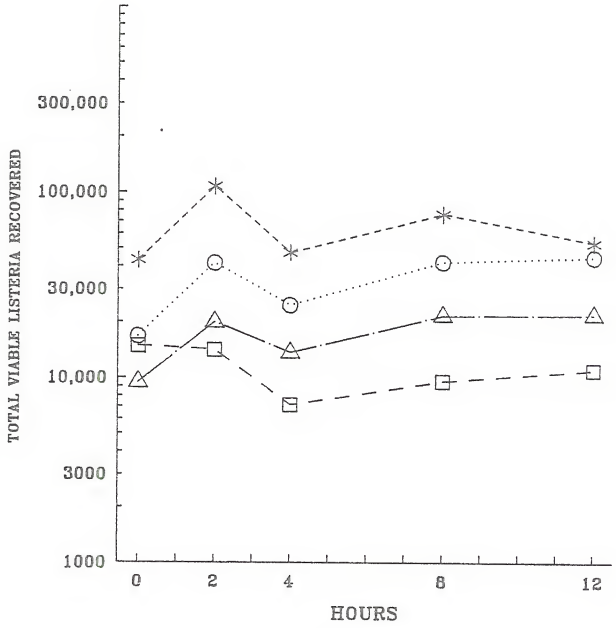


Figure 12. Surviving intracellular Listeria as a function of the length of coincubation. Each point (logarithmic scaling) is the average total viable intracellular Listeria recovered from 3 preparations at the times indicated. Listeria were isolated from infected cells obtained from 10V (*), 2.5V (O), NH_4Cl (□), or phosphate buffer (△) -treated mice.

intracellular Listeria increased for the buffer treated, 2.5V and 10V mouse PEM by 111%, 148% and 150%, respectively. The ammonium chloride group showed a slight decrease over the first 2 hr. The control cells showed decreases of 32%-50% from t_2 to t_4, while the 2.5 and 10V had decreases of 41 and 57%, respectively. Over the period from 4-8 hr post-phagocytosis, levels of Listeria increased for all groups with increases of 34% and 57% in the ammonium chloride and phosphate buffer groups, and 63% and 71% in the 2.5 and 10V, respectively. Over the remaining 4 hr period (t_8-t_{12}), the increases were smaller with only 1%-7% for the controls and 15%-30% for the 2.5 and 10V, respectively.

Surprisingly, the overall change in the levels of viable intracellular Listeria was smaller for the 10V group than for the phosphate buffer (25% vs 130%), while the 2.5V group had an overall increase of 170%. The ammonium chloride group had a net decrease of 26% from the starting level. Although the net changes seem small, levels of bacteria immediately after the 20 min phagocytosis period were 180% and 450% higher in the 2.5 and 10V cells than in the buffer group, and 200% to 250% higher by t_{12}. The ammonium chloride group had levels initially 147% higher than in buffer PEM, but levels were 50% lower by t_{12}.

Cell lysis following intra-PEM proliferation of non-killed Listeria may have had an effect upon the numbers of PEM available for analysis (e.g. less cells and therefore the appearance of less viable organisms at any time point).

An analysis of the effect of incubation of freshly harvested PEM in the antibiotic-free medium for the same amount of time as in the killing assay showed that there were small but insignificant decreases in cellular viability with time (Figure 13). Microscopic analysis of the cell populations collected from the test suspensions indicated that over the period of the incubation of Listeria with the PEM, the bacteria were replicating within the phagosomes and spreading through the cytoplasm. When cell integrity was compromised, the cells lysed and bacterial contents were released to reinfect cells in the suspension. The average decrease in PEM populations from the starting levels due to removal of cellular aliquots for sampling was predicted to be 25, 38 and 50% in the 4, 8 and 12 hr samples, respectively. At t_4, the percentage loss was 62% for the 10V group and 74% for 2.5V cells (Figure 14); the total number of PEM in the ammonium chloride group was decreased by 60% and in the buffer group by 43%. At t_8, the controls were 70% and 80% lower than at the start, and the 2.5 and 10V groups were 88% and 73% lower, respectively. By t_{12}, all groups had decreased levels of PEM on the order of 95%-98%.

Discussion

Studies of the mechanisms of host resistance to the pathogenic gram-positive bacteria Listeria monocytogenes have been performed for over 30 years. In early studies, Murray (1955) noted that the minimum lethal dose of Listeria

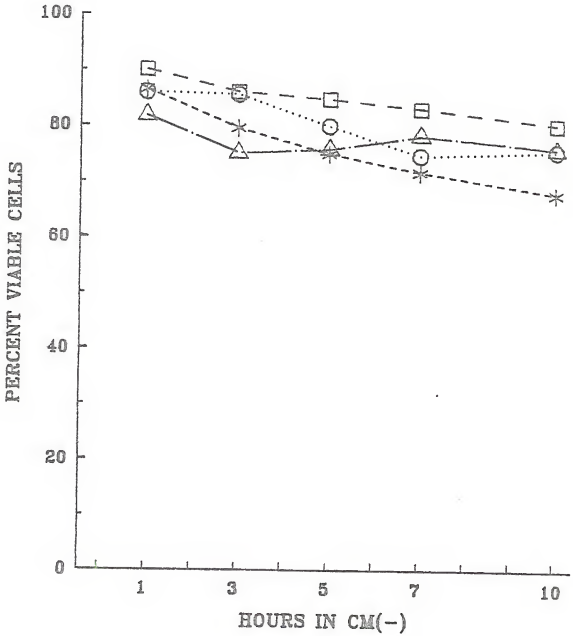


Figure 13. Macrophage viability as a function of time spent in the CM(-) medium. Viability of cells from 2.5V (*), 10V (O), NH_4Cl (□), or buffer-treated (Δ) mice measured using trypan-blue exclusion.

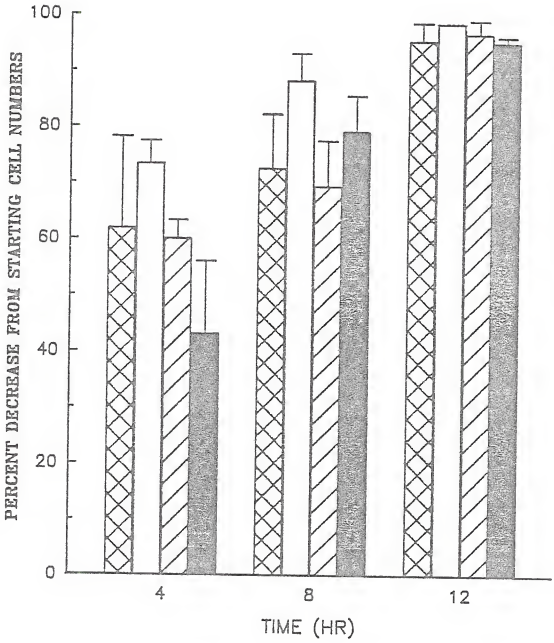


Figure 14. Percentage loss of macrophages during incubation with viable Listeria. Bars represent the mean loss (± SD) of cells from the start at the times indicated. Each bar is the average obtained from a minimum of 5 cell aliquots per treatment per time period. Bars represent 10V, 2.5V, NH_4Cl and phosphate buffer (left to right within each cluster).

was decreased subsequent to passage through a host. Mackaness (1962) explained that this enhancement of virulence was the result of adaptation to intracellular conditions following parasitization. Mutants were selected for survivability within phagocytes and so the lethal dose was subsequently altered after each passage. Mackaness stressed that the pathogenicity of Listeria for mice was totally dependent upon the ability to parasitize mononuclear cells.

The early studies by Mackaness (1962 and 1964) went into great detail in measuring the clearance of IP and intravenous (IV) doses of virulent Listeria. Within 2 hr post-IP injection, the recovered levels of Listeria in the peritoneum were decreased by 6 log levels. In the present study, the control mice injected with 2.4×10^4 organisms had no detectable levels of Listeria by 4 hr post-infection. However, as Mackaness and others have noted (Ruttenberg and van Noorle Jansen, 1975; Saito et al., 1986), the removal of the bacterial challenge did not occur solely in the peritoneal cavity. Direct parasitization of hepatic tissue (including Kupffer cells) and entrapment of infected phagocytes in organs rich in reticuloendothelial elements (e.g. spleen) appear to be the primary modes for spread of the bacteria from the injection site (Kongshavn, 1986).

Many studies using IV routes for Listeria exposure have shown that greater than 90% of the inoculum could be recovered in the host liver within 10 min (Kongshavn and Skamene, 1984), with the remainder appearing in the spleen.

However, following an IP injection, the growth dynamics of Listeria were different. The study by Saito and his colleagues (1986) indicated that detectable levels of Listeria were first found in the liver 24 hr after infection with 2 x 10^6 organisms, and were still detectable for up to 1 week. In the present study, the control mice had measurable levels of Listeria 24 hr after infection, with a maximum number attained by 48 hr. By the third day of infection, the livers of control mice still had assayable levels of the bacteria, but these levels were at or below those at 24 hr.

The analysis of splenic Listeria after IP dosing of 5 x 10^4 organisms resulted in no detectable bacteria for up to 6 days past the initial infection (Ruttenberg and van Noorle Jansen, 1975). By doubling the infectious dose to 1 x 10^5, splenic levels of $>10^6$ bacteria per gram of tissue were observed. In the study reported here, bacteria were detected in the spleens of control mice 24 hr after infection with a dose of only 2.4 x 10^4 Listeria, and increased slowly through the remaining 2 days of study. Levels of Listeria were consistently on the order of 10^4/g spleen; this suggested possible differences due to bacterial virulence.

The resulting clearance of the Listeria from the peritoneum, liver, and spleen in the 3-5 days following infection or the logarithmic increases in bacterial numbers over the first 2 days have been documented in many studies (Tripathy and Mackaness, 1969; Cheers et al., 1978; Skamene and Kongshavn, 1983). The initial trapping was by resident

macrophages such as splenic macrophages, PEM or Kupffer cells. Treatment with carageenan, dextran sulfate, or silica preferentially depleted macrophage function (Allison et al., 1966; Catanzaro et al., 1971; Sadarangani et al., 1980; Newborg and North, 1980) and depleted the numbers of circulating monocytes resulting in fulminating Listeria infections (Mitsuyama et al., 1978).

The studies with the 2.5 and 10V treated mice yielded patterns of Listeria growth in the liver and spleen similar to that seen with carageenan-treated mice. This suggested that the decrease in resistance to Listeria was due either to a vanadium-driven depletion of macrophage function alone or in combination with a decrease in the recruitment of monocytes to the site of infection. The latter point is supported by increasing levels of bacteria after the initial rise in bacterial levels 24-48 hr post-infection. Had recruited macrophages been present, the levels of bacteria should have declined as was apparent with the control mice.

A further contribution to the observed decreased resistance could be due to the nutritional status of the infected mice. The significant loss of body weight in the vanadate-treated infected mice might place these hosts in a catabolic state. The impact of such a metabolic condition on the resistance to Listeria should be further examined. However, the sharp decline in body weight appeared to be secondary to the more rapid onset of infection in the

vanadate-treated mice and was not due to vanadium exposure itself (Cohen et al., 1986).

Although recruited monocytes are converted into activated macrophages at the infection site, the role of activated cells in listeriosis (North, 1970; Ratzan et al., 1972) has come into question (Czuprynski et al., 1983). It was suggested that the monocytes, resident macrophages, and polymorphonuclear leukocytes (i.e. neutrophils), and not monocyte-derived activated macrophages, were the major cellular components involved in the latter phase of bacterial removal (Czuprynski et al., 1984; Lepay et al., 1985).

The role of T-cells has also been well documented. Following bacterial processing by the resident macrophage, naive T-cells were activated to release leukotactic lymphokines (McGregor and Koster, 1971; Lane and Unanue, 1972; North, 1973; Takeya et al., 1977; Campbell, 1979). Thus the early stages of bacterial removal were considered natural (non-immunologically induced) resistance while the latter phases were the result of a specific acquired immunity.

The known inhibitory effects of vanadium on DNA metabolizing enzymes such as thymus terminal deoxynucleotidyl transferase and DNA polymerase (Sabbioni et al., 1983) and on the mitogenic responsiveness and proliferation of T- and B-lymphocytes (Ramanadham and Kern, 1983; Marini et al., 1987) may result in a depletion of proliferating T-cells. In an earlier study (Cohen et al., 1986), vanadium pretreatment resulted in a dose- and time-dependent thymic atrophy.

The loss of T-cell participation in the mechanism of resistance to listeriosis was not addressed by the present study, but would have significant consequences in the ability of the host to survive infection. This latter point requires future study to further clarify how vanadium interrupts murine resistance to viable Listeria monocytogenes.

The effective removal of Listeria as a potential lethal threat was therefore dependent upon engulfment and intracellular killing of the pathogen. Mackaness (1962) determined that the doubling times for intracellular Listeria in resident liver and spleen macrophages were 5.1 hr and 4.8 hr, respectively. Davies (1982, 1983) performed detailed kinetic studies that implied that killing began 3 min after contact of the bacteria with activated phagocytes. However, when studies were performed with normal resident macrophages, the loss of bacterial viability for the same period was 3-fold lower. These two studies suggest that in both cases, phagocytosis was the rate controlling step of the biphasic process.

Several studies have measured the phagocytic activity of mouse PEM, but differences in the length of the infection period and the presence or absence of lymphocyte supernatants prevented a uniform interpretation. In vivo studies using IP injection of the organisms resulted in levels of uptake on the order of 24%-36% (percent positive PEM) after 1 hr of coincubation (de Heer et al., 1980; Leijh et al., 1984). Using a nonopsonized bacteria and resident cells,

Hashimoto et al. (1986) found a 25% uptake within 30 min. Wilder and Edberg (1973) found levels ranging from 18%-20% to 30%-35% after 2 hr, depending on the virulence and coat texture (rough vs smooth) of the Listeria variant. These latter studies relied upon PEM affixed to glass surfaces, a condition known to alter phagocytic and killing activities (Lazdins et al., 1980; Leijh et al., 1981). Czuprynski et al. (1984) indicated that resident PEM maintained in suspension with a 5-fold higher level of bacteria had a phagocytic index of 24%-30% within 1 hr. In the present study, control PEM had phagocytic indices of 22%-27% after a 2 hr coincubation period. However, vanadium-treated mice showed a dose-dependent lower uptake of Listeria and an unusual decrease in the index with an increase in the length of coincubation.

As noted earlier, during the process of phagocytosis there is a great increase in cellular respiratory activity, glycolysis, and oxidation of glucose via the hexose monophosphate shunt. As outlined in Chapter IV, studies of glucose-6-phosphate dehydrogenase in the harvested PEM showed that pretreatment with vanadium resulted in a dose-dependent decrease in the activity of this enzyme crucial to shunt function. The subsequent decreases in the activities of glutathione reductase and peroxidase and increases in intracellular oxidized glutathione might have resulted in further alterations in normal cellular function.

With a disturbance in proper energy metabolism and the respiratory burst, the ability of the PEM to engulf membrane-bound Listeria would also be affected (Karnovsky, 1961). This might explain why there was a significantly lower phagocytic index using PEM from vanadium-treated mice; the decreased uptake after only an additional hour of coincubation is more difficult to explain.

The possibility that toxicity from a build-up of GSSG might affect cell function has been demonstrated (Jacob and Jendl, 1966). Direct effects on phagocytosis and hexose monophosphate shunt activity have also been shown to occur with disturbances of the GSH:GSSG balance (Holmes-Gray et al., 1971). Although the phagocytic indices in PEM from both the 2.5 and 10V-treated mice were decreased between the first and second hours of coincubation, the decline was not statistically significant. A more detailed study of the PEM from the vanadium-treated mice would be required to determine if GSSG levels were altered (e.g. increased) during the course of the coincubation.

Based upon the observed disturbances in peroxidase activity, the ingestion of Listeria (which stimulates superoxide formation) might result in a buildup of cellular peroxides which can distub macrophage function and inhibit killing. However, this should have resulted in a greater number of infected PEM (or a level no less than the percentage of positive PEM obtained after 1 hr) or PEM containing greater numbers of organisms (which was not observed).

The last possibility is that those PEM initially infected with Listeria did not efficiently kill the organism and replication continued until the PEM succumbed and were lysed. An intracellular generation time of 90 min has been reported (Bennedsen et al., 1977) and earlier-infected PEM might be killed within 2 hr. This would result in a lowered number of "earlier-infected" PEM and a subsequent reinfection of the PEM suspension. A reinfection could explain why the population patterns of intracellular Listeria did not show any recognizable trend over a 2 hr test period. The reinfection process could also lead to the appearance of lowered phagocytic indices due to a diminishing population of phagocytically-active cells.

If the lowered number of Listeria-positive cells was the result of ineffective killing in addition to a lowered phagocytic activity (the result of enzyme inhibition), then PEM from vanadium-treated mice should show similar dose-dependent decreases in intracellular killing over the 2 hr period. In all the treatment groups (except for the ammonium chloride mice), PEM showed little killing in this period, with intracellular bacterial levels increasing from 111%-150% for the phosphate control and the vanadium-treated mice. The trend in bacterial numbers over the early period was similar to that observed by Ratzan et al. (1972) and Karnovsky et al. (1975b).

This increase in Listeria populations reflects the above cited intracellular doubling within 90 min of

infection. An early study indicated that within 1 hr, intracellular populations could increase from an average number of 2 organisms to 17 (Simon and Sheagren, 1971). At a level of infectivity closer to that used in this study, a maximum intracellular population of 7 bacteria/PEM was reached after 2 hr (Wilder and Edberg, 1973). These latter authors indicated that although intracellular killing was a continuous event, surviving Listeria continued to replicate over the course of 6 hr. Studies using a virulent strain of Listeria showed a similar increase in bacterial levels over the first 2 hr of coincubation with a steady decline up to 8 hr. After this, bacterial numbers increased for the next 16 hr. Harrington-Fowler et al. (1981) reported intracellular increases of 177% as compared with the starting levels within 4 hr, dropping to 131% by 6 hr (Harrington-Fowler and Wilder, 1982).

Few studies have followed the patterns of intracellular killing longer than 2 hr, and even fewer past 6 hr. Wilder and Edberg (1973) monitored intracellular killing up to 24 hr, and Cole (1975) assayed for 72 hr. In both of these studies, the population of surviving Listeria began to rise steadily after 8-12 hr of cell infection. This was most likely due to the enrichment of macrophage-resistant organisms and mimiced the observations of Murray (1955) regarding passaging and virulence. However, all these in vitro assays of intacellular killing did not accurately

reflect events in vivo since recruitment of activated macrophages and monocytes was not possible.

The exact mechanism of intracellular killing of Listeria in macrophages has not yet been elucidated. Studies have implied that killing may be dependent on phagolysosomal fusion as in the case of Trypanosoma cruzi (Jones and Hirsch, 1972; Osuna et al., 1986) and Mycobacterium tuberculosis (Goren et al., 1976); both organisms antagonize the fusion process to ensure survival in the cell. Listeria causes delays in the fusion process during the first hours of infection. In macrophages containing dead Listeria within fused phagolysosomes, contributions from lysosomal hydrolytic enzymes could not be demonstrated (de Heer et al., 1980).

Although the role for lysosomal enzymes in the killing of Listeria remains unclear, the possible effects from vanadium exposure might lead to problems in both the phagosome-lysosome fusion as well as the activity of the hydrolytic enzymes. Movement of phagosomes and lysosomes to allow fusion is along microfilaments within the cytoplasm (Reaven and Axline, 1973; Malawista, 1975; Kielian et al., 1982). Exposure of cells to vanadate led to dissociation of microfilaments and microtubules and displacement of nuclei and other organelles in uninfected cells (Wang and Choppin, 1981). These authors noted that the possible mechanism of vanadate action was via inhibition of the Mg^{2+}-ATPase

activity of a microtubule-associated protein thought responsible for tubule motility, dynein (Gibbons et al., 1978) .

The presence of polyanions (Goren et al., 1984) and certain negatively charged metal complexes (Weiser, 1939; Goren et al., 1987) have also been shown to block fusion and diminish intracellular killing of yeast and bacteria. Vanadate has been shown to polymerize under specific pH conditions to yield the polyanion, decavanadate (Chasteen, 1983; Phillips et al., 1983). Thus, although the roles of phagosome-lysosome fusion and the release and activity of hydrolytic enzymes in the killing of ingested Listeria have not yet been fully defined, a role for vanadium inhibition in these processes remains speculative.

Other possible mechanisms for intracellular killing of Listeria via the oxidative action of hydrogen peroxide and/or the toxic action of activated halogens such as the chlorinium or iodinium ion have been proposed. The latter mechanism requires the presence of myeloperoxidase (MPO), an enzyme found at extremely low levels in mature resident and activated mouse PEM (van Furth et al., 1970; Nichols and Bainton, 1975). However, promonocytes, monocytes, and polymorphonuclear leukocytes (i.e. eosinophils and neutrophils) contain adequate levels of this lysosomal enzyme. Saito et al. (1986) and Czuprynski et al. (1983 and 1984) showed that these non-PEM cells were recruited to the foci of infection and play an important role in the resistance to Listeria.

The ingestion of damaged neutrophils and eosinophils during the inflammatory response may provide a source of MPO for resident PEM, but several studies (Biggar et al., 1976; Harrington-Fowler et al., 1981; Harrington-Fowler and Wilder, 1982) indicated that peroxidase played little or no role in resident, inflammatory, or immune macrophages during the killing of Listeria. This was best shown using stimulated cells prepared from thioglycollate-treated mice; these had very high levels of peroxidase activity but were weakly listericidal. If MPO had been shown to play a major role in the intracellular killing of Listeria, then the present study predicts that this peroxidase might be affected by the presence of vanadate ions.

The use of oxygen-dependent mechanisms for intracellular killing of Listeria seems less likely in an oxygen-poor environment such as the peritoneal cavity. Resting PEM treated with zymosan or phorbol myristate acetate (PMA) produced little if any superoxide anion or hydrogen peroxide (Johnston et al., 1978). However, if the cells were stimulated for prolonged periods with these agents or the cells were obtained from Listeria-infected mice, superoxide production and release was greatly enhanced. Weinberg and Misukonis (1983) suggested that the increase in PMA-induced chemiluminescence and superoxide production was the result of increased synthesis of NADPH oxidase within the cell (as opposed to an increasad sensitivity or numbers of receptors for the triggering agent).

Saito and his colleagues (1986) performed similar studies using mice infected with Listeria or Mycobacterium intracellulare and found that PEM from Listeria-infected mice did not respond as well to PMA as determined by both the time until onset of response and the magnitude of response. Hashimoto et al. (1986) stated that although the process of ingestion of Listeria was adequate to trigger superoxide production by PEM (Godfrey and Wilder, 1984), the levels released were only slightly greater that when the host was pretreated with peptone, a weak immunopotentiator (Nathan and Root, 1977).

In vitro studies using unstimulated resident and PEM from Cornyebacterium parvum-treated mice indicated that infection with Listeria resulted in a weak production of superoxide and hydrogen peroxide by the former although the listericidal activity of these cells was greater than observed with the immune-boosted cells. Biggar et al. (1976) indicated that the production and release of superoxide anion and hydrogen peroxide did not correlate with antilisterial activity, and suggested that other as yet unidentified oxidative mechanisms may play a role in the killing process.

Although the role of oxygen metabolites in intracellular killing of Listeria has not been clarified, vanadate may play an inhibitory role. The studies of nitroblue-tetrazolium reduction in vitro (Chapter IV) indicated that resident PEM from vanadium-treated mice had significantly

lowered capacities to (1) phagocytize the opsonized antigen (zymosan) and (2) produce and release superoxide anion into the phagosomes. A recent study (Lison et al., 1988) indicated that the incubation of vanadate with activated PEM for 15 min had no effect upon superoxide production. However, cells in an activated state already have increased levels of cellular enzymes and activities so that the addition of vanadium might have had less overall impact than observed in the resting cells.

Superoxide production studies using metals such as platinum (Sodhi and Gupta, 1986), cadmium and lead (Hilbertz et al., 1986) have shown that macrophages exposed to these metals displayed increased, unaltered, and decreased responsiveness, respectively for each metal, to zymosan or PMA. Although the oxidation of NADPH and the depressed hexose monophosphate shunt activity by vanadate treatment would contribute to decreased oxygen conversion to superoxide anion, a direct analysis of NADPH oxidase activity in cells from vanadium-exposed mice may clarify this portion of the immunoinhibitory mechanism.

Resident mouse PEM possess minimal listericidal activity (Czuprynski et al., 1984) and this likely contributed to the initial increases in Listeria levels in the intracellular killing study reported here. It is likely that the killing systems inherent to the resident PEM were only partially effective against the Listeria and became exhausted during the active infection. The early removal of

Listeria from the peritoneal cavity might have been the result of activity in a specific bactericidal PEM subpopulation. However, this population was eventually overwhelmed by unrestricted growth of Listeria in nonactive subsets (Rice and Fishman, 1974; Lee et al., 1981; Harrington-Fowler and Wilder, 1982). The latter authors also proposed that killing by resident PEM might be due to bacterial factors associated with growth characteristics. Still others have suggested that non-oxidative mechanisms such as an inherent anti-Listeria factor produced by resident PEM (Dumont, 1978; Klebanoff, 1980b) or lysosomal cationic peptides may aid in Listeria destruction (Patterson-Delafield et al., 1980; Lehrer et al., 1981).

The presence of vanadium in the host and probably in the PEM complicates determining the exact mechanism of decreased resistance to Listeria. Although in the control mice limited Listeria killing was observed, the PEM from vanadium-treated mice displayed similar trends in killing but the absolute numbers of surviving organisms was higher (in a dose-related manner) throughout the assay. The present study indicated that these PEM had lower phagocytic indices, and higher levels of Listeria in the cells reflected a decreased ability to eliminate this facultative intracellular parasite.

CHAPTER IV

GLUCOSE-6-PHOSPHATE DEHYDROGENASE, GLUTATHIONE REDUCTASE, AND GLUTATHIONE PEROXIDASE ACTIVITIES, NITROBLUE TETRAZOLIUM REDUCTION, AND GLUTATHIONE LEVELS IN RESIDENT PERITONEAL MACROPHAGES OF MICE FOLLOWING 6 WEEKS OF VANADIUM EXPOSURE

Introduction

The intracellular biochemistry of the cells of the immune system has been studied for many years. A detailed characterization of the enzymology involved in the production of energy and in the intracellular killing processes of leukocytes varies depending on the particular cell under study. Much information has been published regarding these functions in polymorphonuclear leukocytes (PMN) such as eosinophils and neutrophils (Sbarra and Karnovsky, 1959; Iyer et al., 1961; Sbarra et al., 1971; Gabig and Babior, 1981). Less information is available regarding the mechanisms in alveolar macrophages (PAM) (Pearsall and Weiser, 1970; Vogt et al., 1971), Kuppfer cells, or peritoneal macrophages (PEM) (Johnston et al., 1978; Wachsmuth and Wust, 1982). The animal source of the cells also has an effect upon the amount of information available (Pavillard and Rowley, 1962; Rossi et al., 1975). The mouse resident PEM model system used in the studies reported here has only recently received more attention (Simmons and Karnovsky, 1973; Cohn, 1978; Lazdins et al., 1980; Karnovsky et al., 1982; Nibbering et al., 1987).

Many of the biochemical activities elucidated in one type of leukocyte apply to all cells of this class. However, the location of the cell in the host often has a profound influence on some functions. A comparison of the enzymatic profiles of mouse alveolar and peritoneal macrophages reveals differences in both the mechanisms of energy production as well as activated oxygen metabolite formation and/or detoxification. The former relies primarily upon mitochondrial oxidative phosphorylation for the production of energy for phagocytosis, while PEM rely primarily upon glycolysis and the hexose monophosphate shunt (Rossi et al., 1975). A second difference is in hydrogen peroxide destruction; while PAM utilize catalase to form molecular oxygen and water, PEM are deficient in catalase and rely heavily upon glutathione peroxidase (GSHPX) (Simmons and Karnovsky, 1973). Finally, PAM contain the halogen-activating enzyme myeloperoxidase (MPO) which aides in the destruction of ingested microorganisms. The PEM are nearly devoid of MPO (van Furth et al., 1970) and so must rely upon ingestion of MPO-bearing cells (i.e. eosinophils) or use pinocytosis to obtain the enzyme from the surrounding milieu (Klebanoff, 1980b).

While information regarding cellular functions has been expanding, reports of the effects of immunotoxicants such as drugs and metals on these activities have also been increasing. As with most toxicological studies, the early studies presented the results in the standard dose and observed

response manner; few studies attempted to elucidate possible mechanisms of toxicity.

Of all the metals, cadmium has been the most extensively studied for effects on the energy producing pathways of macrophages (Castranova et al., 1980; Hilbertz et al., 1986). The work by Mustafa et al. (1971a, 1971b) provided detailed mechanisms of the inhibitory effect of cadmium in PAM. This group indicated that cadmium ions were capable of inhibiting mitochondrial oxygen uptake and of uncoupling oxidative phosphorylation, thus depriving the cells of the primary source of energy production. Inhibition occurred at the level of NADH- and succinate dehydrogenase activity (Mustafa and Cross, 1971) as well as in the activity of ATPase. The former was deemed to be the result of cadmium binding to active site functional thiol groups; the mechanism for ATPase inhibition was less clear because many other divalent metal ions tested (iron, copper, zinc, cobalt, mercury, and tin) were also inhibitory.

Mustafa et al. (1971b) speculated that metal binding to PAM outer or mitochondrial membranes might cause alterations in compartmental organization. In addition, the presence of metals might have enhanced the labilization of lysosomal enzymes which resulted in intracellular damage. Other metals, such as beryllium, silica and lead have since been shown to cause an increased release of lysosomal proteases which resulted in altered cell function (Gardner, 1984). The metal is ingested in the particulate form and disrupts

phagolysosome membrane integrity so that the organelle ruptures. Proteolytic enzymes are released along with the metal particles and both are capable of interacting with cytoplasmic enzymes and mitochondria.

Following the work by Strauss et al. (1969) which linked the function of cytoplasmic glutathione reductase (GSSG-R) to the process of phagocytosis, energy production and oxygen metabolite formation/destruction, the role of glutathione and glutathione-related enzymes in leukocytes received more interest. Jacob and Jandl (1966) had shown earlier in erythrocytes that glutathione status was critical to the function of the hexose monophosphate shunt (HMS) and glucose-6-phosphate dehydrogenase (G6PDH) activity. Holmes-Gray et al. (1971) demonstrated that interference in the intracellular ratio of reduced (GSH) and oxidized glutathione (GSSG) not only affected the activity of GSHPX and GSSG-R, but also disturbed oxygen uptake and activation (using nitroblue-tetrazolium [NBT] reduction) as well as phagocytic activity. Serfass and Ganther (1975) showed that the dysfunction of glutathione-dependent enzymes affected energy production for phagocytosis and overall macrophage microbicidal activity.

Little is known of the role of immunotoxicants in this particular area of study; investigations dealing with selenium deficiency are reported, but these are in deference to the essential role of the metal in the function of GSHPX. There is limited information on glutathione status and

activities of glutathione-dependent enzymes in mouse resident PEM (Murray et al., 1980; Rouzer et al., 1981; Karnovsky et al., 1982).

The exposure of PEM to vanadium has been shown to result in alterations in phagocytic activity (Cohen et al., 1986) and chemilumiscence/superoxide formation in vitro (Wei and Misra, 1982). Other studies have described morphological alterations following vanadium exposure (Waters and Gardner, 1975) and alterations in lysosomal enzyme activity (Waters et al., 1974). The effects of vanadium on several intracellular enzymes crucial to PEM energy production for phagocytosis and for oxygen processing have either not been fully investigated or reports presented conflicting results (Menon et al., 1980; Nour-Eldeen et al., 1985; Ramasarma et al., 1981). Kinetic studies of select enzymes have been performed in vitro in an attempt to describe possible mechanisms of inhibition within macrophages. Mechanisms for vanadium entry into non-macrophage cells (Macara et al., 1980; Heide et al., 1983) and binds intracellular ligands (i.e. glutathione) and enzymes (Nechay et al., 1986) have been postulated.

Therefore, the purpose of the present study was to determine the possible inhibition of certain enzymes used in oxygen activation and, in part, for the production of energy for phagocytosis, as well as effects on the status of their respective substrates following host exposure to vanadate.

Materials and Methods

Dosing regimen and peritoneal macrophage preparation

Female $B_6C_3F_1$ mice were dosed with vanadate or control solutions for 6 weeks, and two days after the last treatment the mice were sacrificed and the PEM were collected as described in Chapter III. The pooled PEM suspension was pelleted and brought up in a final volume of 3 ml of a 2:1 (v/v) solution of HBSS:TE (triethanolamine buffer, 0.05 M, pH 7.5). The final cell concentration was adjusted to 10^7 PEM/ml HBSS:TE and this solution was divided into two equal volumes. One solution was immediately frozen in a dry ice:acetone system for later use in the GSHPX assay, and the other was frozen and thawed three times for use in the assay of G6PDH and GSSG-R activities. After freeze-thawing, the cell suspension was centrifuged to pellet the sheared membranes. The membranous pellet was removed and examined under a microscope to confirm the effectiveness of the lysing procedure. The supernatant was transferred to a test tube held on ice and then immediately used for the enzyme assays. On each of the 4 days of this experiment, 3 mice per treatment group were sacrificed until a total of 12 mice per treatment were analyzed.

Assay of glucose-6-phosphate dehydrogenase activity

The method of Lohr and Waller (1965) as modified by Vogt et al. (1971) was used to analyze the activity of the G6PDH in the PEM cytolysate (Figure 15). All concentrations

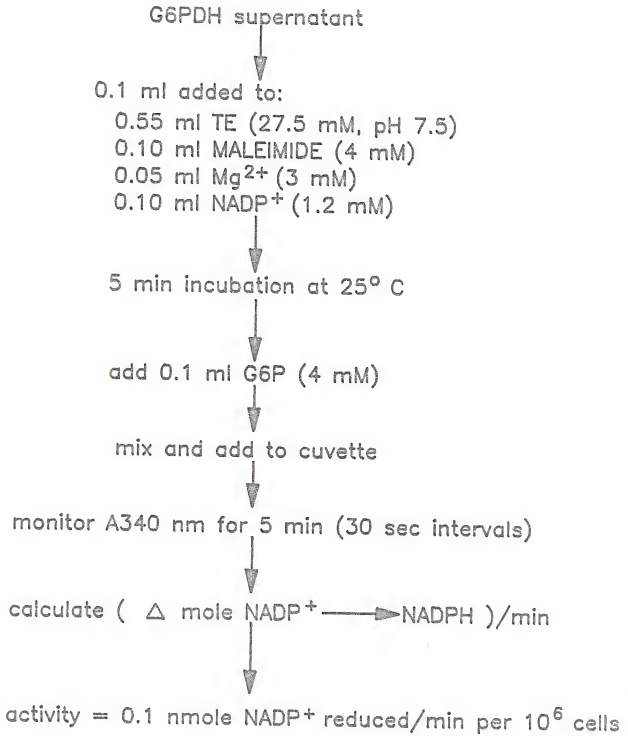


Figure 15. Outline for assay of glucose-6-phosphate dehydrogenase activity in macrophages. All values listed were final concentrations in the 1 ml reaction system.

listed were the final concentrations in the assay system. To 0.55 ml of TE buffer (final concentration 27.5 mM in assay mixture), 0.1 ml of aqueous maleimide (Sigma Chemical Co., 4 mM), 0.05 ml of $MgCl_2$ (3 mM), 0.1 ml of $NADP^+$ (1.2 mM in 1% $NaHCO_3$), and 0.1 ml of the lysate were added. After 5 min incubation at 25°C, 0.1 ml of glucose-6-phosphate (4 mM) was added. The contents were mixed and transferred to a 1 cm quartz cuvette in a Perkin-Elmer double beam spectrophotometer (Model Lambda 3). The reference cell contained all components excluding the $NADP^+$ and the cytolysate.

The absorbance at 340 nm was measured every 30 sec for a period of 5 min. From the change in the absorbance/min, the total moles of NADPH formed/min was calculated using the NADPH extinction coefficient value (6.317×10^3 M^{-1} cm^{-1}). The unit of activity was defined as 0.1 nmole of NADPH formed/min per 10^6 PEM. This definition was modified to express the activity in terms of total protein content in the sample. The assay was repeated 5 times for each treatment cytolysate.

Assay of glutathione reductase activity

The method of Racker (1956) was used to assay GSSG-R activity in the cytolysates (Figure 16). To 0.55 ml of water, 0.05 ml KPO_4 buffer (1 M, pH 7.6), 0.1 ml bovine serum albumin solution (BSA, 0.1% [w/v] in 0.1 M KPO_4 buffer), 0.1 ml NADPH (0.1 mM), and 0.1 ml of lysate were added. After gentle mixing of the solution to avoid forming

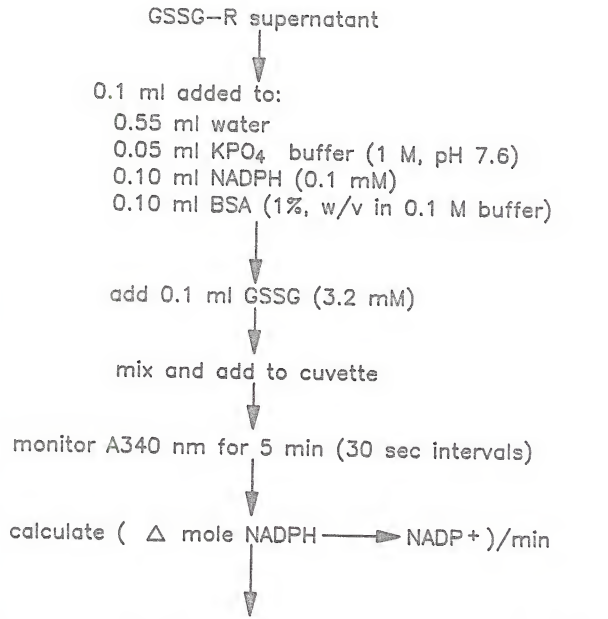


Figure 16. Outline of assay for glutathione reductase activity in macrophages. All values listed were final concentrations in the 1 ml reaction system.

bubbles, 0.1 ml aqueous GSSG (3.2 mM) was added, the solution was mixed and transferred to the cuvette. The reference cell contained all reagents but the lysate and NADPH. The decrease in absorbance at 340 nm was monitored every 30 sec for a period of 5 min. The data obtained was analyzed as above with the G6PDH. A unit of activity was defined as 1.0 nmole NADPH oxidized/min per 10^6 PEM. Each treatment cytolysate was analyzed 5 times.

Assay of glutathione peroxidase activity

The modified method of Paglia and Valentine (1967) by Lawrence and Burk (1976) was used to assay the GSHPX activity in the cytolysates (Figure 17). To 0.71 ml of a solution containing NaN_3, GSH, and Na_2EDTA (1 mM, each in pH 7, 50 mM phosphate buffer), 0.05 ml of bovine mucosa GSSG-R (Sigma, 1 unit), 0.1 ml of NADPH (0.2 mM), and 0.1 ml of lysate were added. After 5 min incubation at 25°C, 0.04 ml of aqueous cumene hydroperoxide (1.5 mM) was added and the solution was transferred to a cuvette. The reference blank contained all reagents but the lysate and NADPH.

The absorbance at 340 nm was monitored every 30 sec for a period of 5 min. The data was treated as above in the GSSG-R experiment. A solution containing HBSS in place of the lysate was also assayed to determine the contribution from spontaneous oxidation of NADPH by the peroxide. This background rate was subtracted from the observed rate in order to obtain the true activity in the cytolysate.

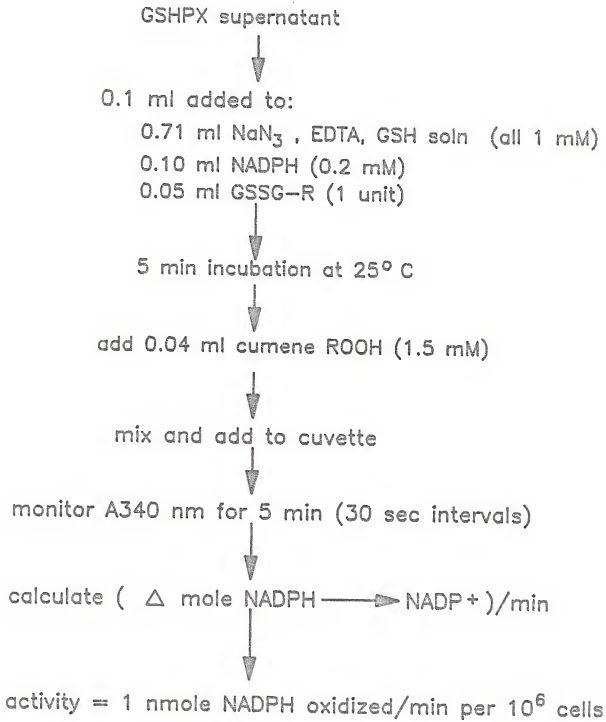


Figure 17. Outline of assay for glutathione peroxidase activity in macrophages. All values listed were final concentrations in the 1 ml reaction system.

Protein determination in cytolysates

The cytolysates were measured for their protein content using the Bio-Rad system (Bio-Rad Laboratories, Richmond, CA). A 0.05 ml aliquot of cytolysate was combined with 2.5 ml of diluted (1:5 of stock) Bio-Rad reagent. The solution was gently mixed and allowed to stand in a stoppered tube for 5 min at 25°C. The absorbance was then measured at 595 nm against a reference cell containing the reagent and HBSS:TE buffer. Each cytolysate was analyzed 5 times. The amount of protein present was calculated from a standard curve of the absorbance vs total protein prepared from the analysis of a BSA solution (in HBSS:TE).

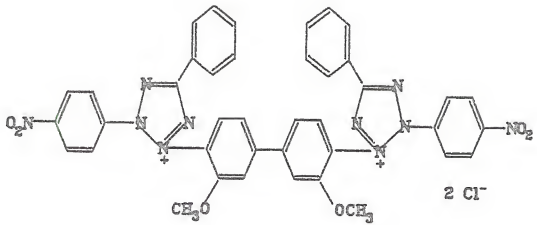
Assay of superoxide anion production via nitroblue tetrazolium (NBT) reduction

The modified method of Wilson et al. (1980) by Godfrey and Wilder (1984) was used to measure superoxide production and release in PEM fed opsonized zymosan. Following the harvesting and population determination steps, the cell suspension was adjusted to 10^6 PEM/ml in complete medium (C-MEM). This medium was composed of 78% minimal essential medium (MEM), 20% heat-inactivated fetal calf serum, 1% L-glutamine, and 1% antibiotic solution (penicillin/streptomycin, Gibco Laboratories, Grand Island, NY). A 0.1 ml aliquot of cells was transferred to a 35 mm x 11 mm coverslip held in a Leighton tube and 0.9 ml of warmed (37°C) C-MEM added. Twenty tubes per treatment were prepared and the tubes stoppered with foam plugs. The tubes were then

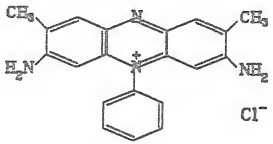
placed in an incubator maintained at a 37°C with a 5% CO_2 atmosphere. The cells were allowed 1 hr to attach to the glass coverslip prior to the addition of fresh medium containing opsonized zymosan and NBT-chloride (Figure 18).

Opsonized zymosan (Sigma) was prepared by the modified method of Johnston et al. (1975) by Damiani et al. (1980). Zymosan particles were suspended in PBS (pH 7.5) at 10 mg/ml, boiled for 60 min, washed 3 times, and resuspended at a final concentration of 50 mg/ml. Particles were opsonized by the addition of 4 volumes of heat-inactivated mouse serum previously collected by heart puncture from untreated mice. The final suspension was adjusted to 10 mg/ml in normal saline to allow for delivery of 1 mg zymosan to the PEM culture (50:1 ratio of particle:phagocyte).

Following the initial attachment phase, the coverslips were rinsed of non-adherent cells with warmed C-MEM. To each tube 0.4 ml C-MEM, 0.1 ml opsonized zymosan, and 0.5 ml NBT (Sigma, 1 mg/ml in C-MEM) were added. The cells were allowed to ingest the particles for 1 hr. The coverslips were then removed and rinsed with warmed PBS. The cells were fixed by immersion in methanol for 30 min. The coverslips were air-dried and cells stained with 0.2% (w/v) safranin O (in 1% acetic acid: Figure 18) for 5 min. The coverslips were rinsed with PBS to remove excess stain, air-dried, and mounted onto slides for counting. A population of 150-200 PEM/slide was examined and PEM that contained



NITROBLUE TETRAZOLIUM SALT



SAFRANIN O

Figure 18. Chemical structures of the nitroblue tetrazolium chloride molecule and of Safranin O stain.

non-diffuse regions of deep blue precipitate were scored as positive for superoxide production.

Assay of macrophage reduced and oxidized glutathione content

The method of Tietze (1969) was employed to measure the levels of oxidized glutathione and the total glutathione (reduced + oxidized forms) in the harvested PEM. The freshly harvested cell suspension was adjusted to 10^7 PEM/ml HBSS:triethanolamine buffer (2:1, v/v, pH 7.5). A 2.0 ml volume was removed and the remaining solution immediately frozen for use in the assay of total glutathione.

The cell suspension was lysed by freeze-thawing as described earlier. To 1.5 ml of cytolysate was added 1.5 ml of 50 mM N-ethylmaleimide (NEM) and the solution was mixed and placed on ice for 1 hr. The content was then transferred to a 20 ml separatory funnel and extracted 5 times with 3 volumes of ethyl ether. The resulting aqueous layer was flushed with nitrogen to remove any remaining ether, the volume of the extract was recorded, and the sample assayed for GSSG.

To 0.8 ml of a 0.75 mM solution of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma), 0.1 ml of aqueous extract and 0.05 ml of NADPH (0.2 mM) were added. All reagents were prepared in 0.1 M sodium phosphate buffer containing 5 mM EDTA, pH 7.5. The mixture was incubated for 5 min at 25°C and then transferred to a cuvette. A 0.05 ml aliquot (1 unit) of bovine mucosa GSSG-R was added and the optical density at 412 nm recorded every 15 sec for a period of

3 min. The reference blank contained the DTNB and NADPH but no enzyme since this led to autooxidation of the indicator. The level of GSSG in the sample was then calculated from a standard curve prepared with stock GSSG extracted as above. Each cytolysate solution was assayed 10 times.

To determine the total glutathione content, the frozen cell suspension was thawed and then treated as outlined earlier to obtain the cytolysate. A 0.1 ml aliquot of the intact lysate was assayed directly in the DTNB system as described above. From the total glutathione level determined in the lysate and from the assayed GSSG levels, the amount of reduced glutathione was estimated.

Statistical analysis

The enzyme activities and glutathione levels expressed in activity units (or nmoles product) per 10^6 PEM (or per mg of total protein), as well as the cytolysate protein levels obtained in cells from vanadate-treated or control mice were analyzed using an analysis of variance (ANOVA) treatment. Duncan's analysis of the means was also applied at $P < 0.05$ and $P < 0.01$ to determine significance among the treatments. The qualitative NBT reduction assay was also analyzed using similar statistical treatments.

Results

The activity of the enzymes G6PDH, GSSG-R, and GSHPX were assayed after the lysing of peritoneal macrophages (PEM) from mice exposed to vanadium for 6 weeks. The

activity of each enzyme was depressed in a dose-dependent manner as compared with that of the controls (Figure 19). The inhibitory effect of pretreatment with 2.5 mg V/kg (2.5V) or 10 mg V/kg body weight (10V) was more severe regarding the dehydrogenase and reductase than with the peroxidase.

As noted in Table 4, when the enzyme activity was expressed as units/10^6 PEM, there was a decrease in dehydrogenase activity due to vanadium exposure. Based upon the known effects of vanadate on cellular protein degradation (Seglen and Gordon, 1981), the cytolysates were assayed for protein content in order to account for any possible contributions to activity from a buildup of non-degraded enzyme. When the data was expressed as units/mg protein present, a similar pattern of depressed activity was observed. Enzyme activity from the 2.5V mice was 59% of the controls while that from 10V mice was only 29%, significantly lower than the controls ($P < 0.01$). There was no significant difference between the activities of either controls.

The studies of GSSG-R activity in the PEM revealed similar results. On an activity/10^6 PEM basis, the activity from the vanadium treated mice was depressed as compared with the controls. Based on the amount of protein present, the 2.5V treatment resulted in significantly lower activities 56% of the control values ($P < 0.05$) while 10V yielded activities 34% of the control ($P < 0.01$). No significant difference between the controls was observed.

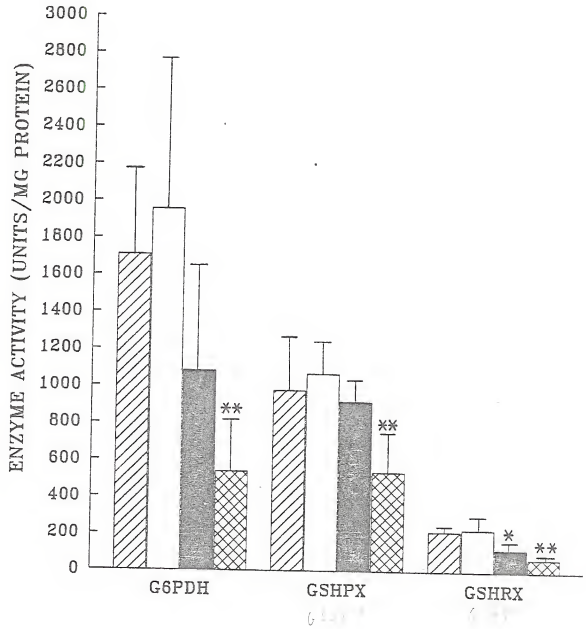


Figure 19. Enzyme activity of the dehydrogenase, reductase and peroxidase in peritoneal macrophages. Each bar represents the mean activity (± SD) from 6 separate assays (each with 5 replicates) using freshly harvested cells. Each cluster represents the specified activity in cells from phosphate buffer, NH_4Cl, 2.5V or 10V-treated mice (left to right in each cluster). Activities significantly different from the controls at $P < 0.05$ (*) and $P < 0.01$ (**) are indicated.

TABLE 4. Mouse peritoneal macrophage enzyme activities and cytolysate protein levels after six weeks of intraperitoneal exposure to ammonium metavanadate.

TRT[a]	ACTIVITY[b] (UNITS/10^6 CELLS)			ACTIVITY[b] (UNITS/MG PROTEIN)		
	G6PDH	GSSG-R	GSHPX	G6PDH	GSSG-R	GSHPX
2.5V	13.77±4.56	1.65±0.44[d]	12.37±2.80	1087.21±610.89	129.77±65.47[d]	924.59±141.02
10V	14.42±2.87	2.06±0.42	14.92±4.64	542.79±289.76[e]	77.14±40.90[e]	542.89±241.62[c]
N^+	20.17±7.17	2.38±0.42	12.45±2.64	1977.24±797.40	235.30±76.09	1072.81±170.02
P	18.78±6.31	2.40±0.45	12.37±2.80	1735.06±464.73	221.90±37.82	982.62±302.80

	CYTOLYSATE PROTEIN LEVELS[f] (mg x 10^{-3})	
2.5V	N = 11	14.11 ± 4.65
10V	10	26.57 ± 12.10[c]
N^+	11	10.69 ± 3.17
P	11	11.98 ± 4.06

[a]Treatments: 2.5 mg V/kg, 10 mg V/kg, NH_4Cl, phosphate buffer.

[b]Units: 1.0 nmole NADPH oxidized/min for GSSG-R and GSHPX, and 0.1 nmole NADPH formed/min for G6PDH. Each value is mean ± SD of 6 separate experiments (5 replicates per cytolysate).

[c]Values significantly different from all others at $P < 0.01$.

[d,e]Value only significantly different from controls at $P < 0.05$ and at $P < 0.01$, respectively.

[f]Mean (± S.D.) in 10^6 PEM from N cytolysates examined.

In the peroxidase assay, the 2.5V pretreatment exhibited its least inhibitory effect. The activity was still lower than the paired controls with a relative activity of 90%. The 10V pretreatment again resulted in a significant depression in enzyme activity ($P < 0.01$). The 10V activity was only 53% of the controls and was significantly lower than even the 2.5V treatment.

The cytolysates were analyzed for their protein content. The protein levels determined using the Bio-Rad system were significantly increased by the 10V pretreatment ($P < 0.01$). Levels of protein in the 10V lysates were 2.2-2.5 times greater than in the control cells, and 1.9 times higher than from the 2.5V mice. The 2.5V regimen increased the level of protein in the PEM 18%-30% above control levels, but this increase was not significant ($P < 0.05$).

The production and release of superoxide anion in PEM from vanadium-treated mice was significantly decreased in a dose-dependent manner ($P < 0.01$). The control resident PEM yielded populations of 41%-45% positive cells while those from mice dosed with the 2.5V treatment had only a 26% positive population. The PEM from mice treated with the highest vanadium dose (10V) were only 18% positive, a value significantly lower than that from both the controls and the lower vanadium dosage (Figure 20).

The analysis of the total GSH pool (oxidized + reduced forms) per 10^6 PEM (Figure 21) indicated that there was no significant difference between the groups as a result of

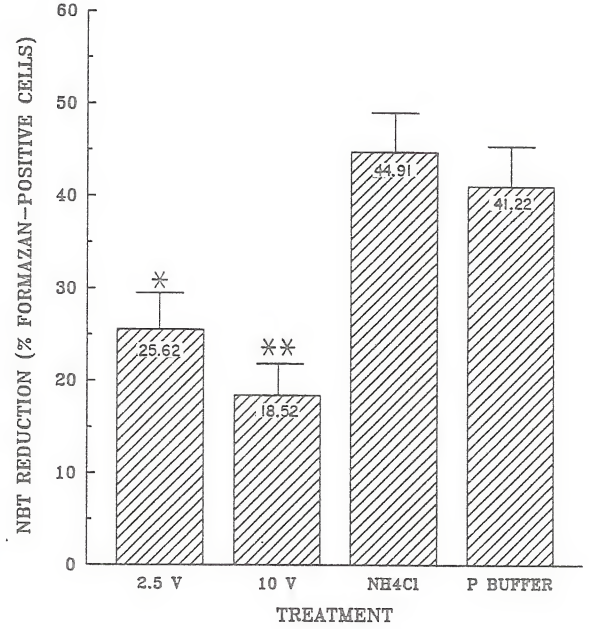


Figure 20. Percentage of positive macrophages using the NBT-reduction assay. Each bar represents the average number (± SD) of positive macrophages observed in 20 preparations/treatment (counts of 150-200 cells/slide). Values significantly different from the controls (*) and from all others (**) at $P < 0.01$ are noted.

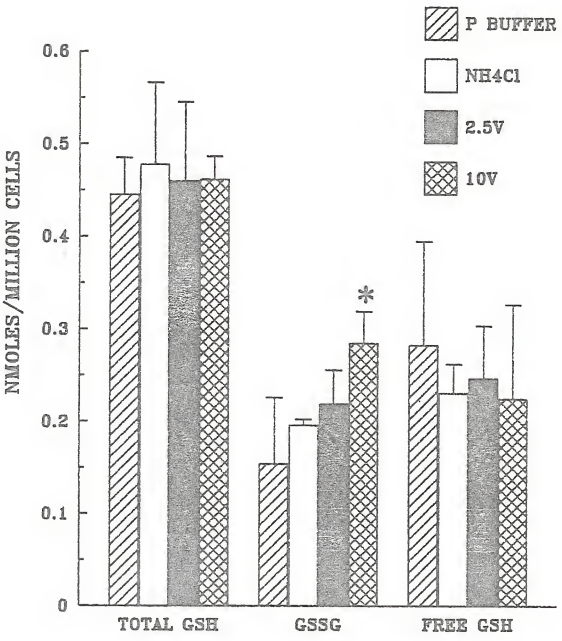


Figure 21. Levels of oxidized and free reduced glutathione and total glutathione (both forms) in peritoneal macrophages. Each bar represents the mean ($\pm$ SD) level of each compound in freshly harvested cells from buffer, NH_4Cl, 2.5V or 10V-treated mice. Values significantly different from controls at $P < 0.05$ (*) are noted.

the 6-week pretreatments. When PEM were assayed for the oxidized form (GSSG), the 10V-treated mice had significantly higher levels (nmoles GSSG/10^6 PEM) than the controls. Although the 10V mice had the highest levels, the amount was not significantly greater compared to that of the 2.5V mice. The 2.5V and 10V treatments resulted in increased levels of 25% and 63% over the paired controls, respectively. Vanadium treatment also resulted in a lower level of free reduced GSH as compared with the controls. Although the difference in GSH levels was not significant ($P < 0.05$), the trend was the inverse of the GSSG results with the 10V mice having the lowest reduced GSH levels; vanadium-treated mice had levels 17% lower (nmoles GSH/10^6 PEM) than the controls.

When the data was analyzed on the basis of lysate protein present, vanadium-treated mice showed significantly lower levels of total GSH as compared to controls. The 10V mice had levels of 24 nmole/mg protein while the 2.5V mice had 28 nmole/mg. The ammonium chloride control had a level of 36 nmole/mg, a value significantly greater than in cells from both vanadium treatments ($P < 0.05$). The phosphate buffer-treated mice data could not be readily interpreted due to problems with the protein analysis. The 10V mice had consistently higher levels of protein in the lysates and so skewed the results lower in both the GSH and GSSG analyses.

The cytolysates were also analyzed to determine if there was any shift in the ratio of levels of GSSG to GSH (Figure 22). The ratios of GSSG to free GSH and of GSSG

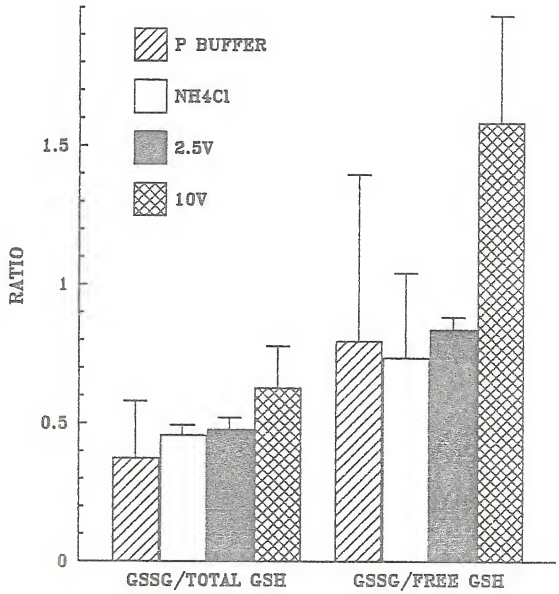


Figure 22. Ratios of oxidized glutathione to the free and total glutathione pools. Details were presented in Figure 21.

to the total GSH pool were increased by the vanadium treatments in a dose-dependent manner, although the increases were not statistically significant ($P < 0.05$). These trends in the ratios obtained were similar from day to day but attempts to pool the data resulted in large standard deviations. The vanadium-treated mice had GSSG levels equivalent to 48%-63% of the total GSH pool compared to 42% for the controls. Compared with the free GSH, the GSSG levels in cells from vanadium-treated mice yielded ratios of 0.84:1 (2.5V) or 1.59:1 (10V) while controls averaged 0.74-0.80:1.

Discussion

Exposure of mice to ammonium metavanadate has been shown to alter their resistance to *Listeria monocytogenes* as well as to depress the phagocytic capacity of their resident PEM (Cohen et al., 1986). The purpose of the present study was to determine if these depressed responses after vanadate pretreatment was due to inhibition of enzymes essential to phagocytosis and killing of pathogens. For this, glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GSSG-R), and glutathione peroxidase (GSHPX) were assayed for activity in harvested murine PEM following vanadate pretreatment for 6 weeks.

In the earlier study, the treatment of mice with vanadate for 6 weeks led to a more rapid onset of 100% mortality when challenged with an IP LD_{20} dose of viable *Listeria*. Similarly, the phagocytic indices of the resident PEM were

depressed in a dose-dependent manner to levels 80%-86% of the controls (Cohen et al., 1986). The process of phagocytosis and the ultimate killing of the pathogen was dependent upon the respiratory burst. This burst, along with increased glycolysis, produced the substrates necessary for energy production for phagocytosis as well as for the uptake of extracellular oxygen. The burst was also responsible for the production of activated oxygen species for intraphagolysosomal killing of pathogens. In the PEM, the production of potent oxygen metabolites was critical since this cell lacks several enzymes for killing activity which are present in other leukocytes (Klebanoff and Hamon, 1975; Gabig and Babior, 1981).

The experimental values for G6PDH activity in the non-elicited, non-activated murine PEM in this study fell into the predicted range for this cell. There are only a few reports which detail the activities of hexose monophosphate shunt (HMS) enzymes in mouse PEM. These studies analyzed the capacity of cultured PEM to oxidize radiolabelled glucose to CO_2 as a determinant of overall shunt activity. Activity ranged from 20-100 nmole CO_2/mg protein per hour, or a maximum of 1.67 nmole CO_2/min (Karnovsky et al., 1982). Lazdins et al. (1980) determined that CO_2 generation was 7-8 times greater in macrophage suspensions than in cultured cells.

In the study presented here, the level of activity of the HMS enzyme was 180 nmole $NADP^+$ reduced/min per mg

protein. However, this analysis was performed in a membrane-less cytolysate system; therefore proteins were not present to be calculated into the denominator of the activity expression. Cohn (1978) reported a total of 0.08 mg protein in a suspension of 10^6 resident mouse PEM. In this study, the average level of protein in control PEM was 0.01 mg. Accounting for this loss of protein, the activity in the controls would then be approximately 22.5 nmole $NADP^+$ reduced/min per mg protein.

Using a different model system, Vogt et al. (1971) measured G6PDH activity in rabbit PAM and reported a value of 4 nmole $NADP^+$ reduced/min per 10^6 PAM; in the present study the value was 1.6-2.6 nmole/min. Karnovsky et al. (1970) reported that PAM had respiration rates and capacities 2-3 times greater than in PEM. The activities in the murine PEM in this study were therefore in an acceptable range for this enzyme.

On the basis of lysate protein present, the treatment of mice for 6 weeks with 2.5 or 10 mg V/kg doses led to a 41% and 70% decrease in G6PDH activity, respectively. The few studies of the effects of metals on macrophage enzymes have been performed with harvested cells after in vitro exposure to metal. Mustafa et al. (1970 and 1971a) used sheep PAM and determined that cadmium inhibited succinate and alpha-ketoglutarate dehydrogenase activities in isolated mitochondria. This group (1971b) later showed that cadmium was able to inhibit electron transport function by binding

to flavin- and pyridine-linked dehydrogenases. Fluharty and Sanadi (1960) and Searls et al. (1961) had demonstrated that cadmium acted as a dithiol binding agent. This suggested that cadmium inhibition of dehydrogenase was due to specific binding of functional dithiol groups on the enzyme.

In Chapter V, vanadate inhibition of yeast G6PDH is reported. Vanadium oxides were known to bind thiol groups to yield thioesters of vanadium (Sakurai et al., 1981; Legrum, 1986). It had also been previously shown that vanadium exposure in vivo resulted in inhibition of glycolytic enzymes such as hexokinase (Climent et al., 1981). With less initial phosphorylation of glucose to glucose-6-phosphate, the HMS activity was expected to decrease. Based upon these effects of vanadium exposure and the earlier cited studies of cadmium and cellular dehydrogenases, the in vivo inhibition of PEM G6PDH by vanadate was not unexpected.

Glutathione reductase (GSSG-R) plays a role as the substrate regeneration enzyme for glutathione peroxidase-mediated detoxification of cellular peroxides. Using guinea pig PMN, Strauss and co-workers (1969) demonstrated the importance of the reductase as an early generator of $NADP^+$ following phagocytic stimulus. This increased GSSG-R activity allowed for enhanced HMS production of NADPH which was necessary for the generation of superoxide anion. The increase in cellular peroxides following spontaneous dismutation stimulated the activity of the glutathione redox cycle and so ultimately, the GSSG-R itself. The increase in

GSSG-R activity was apparent within 15 sec of phagocytic stimulation while the activity of NADPH oxidase did not significantly increase until nearly a minute later. Strauss concluded that GSSG-R was a major participant in the initiation of the HMS to aid in energy production needed for phagocytosis, as well as an enzyme crucial for the removal of activated oxygen metabolites.

The activity of GSSG-R in PMN was determined to be 0.33 nmole NADPH oxidized/min per 10^6 PMN. However, mature macrophages contain less catalase than PMN and so rely more heavily upon the glutathione redox cycle for peroxide destruction. As a result, macrophage GSSG-R activity was 8-10 times greater than in PMN. Gee et al. (1971) determined the activity in rabbit PAM and found it to be 2.6 nmole NADPH oxidized/min per 10^6 PAM. A literature value for GSSG-R activity in non-elicited, non-activated murine PEM was not available. The values obtained in this study were similar to that observed in PAM; GSSG-R activity in controls was 2.1-2.8 nmole NADPH/min per 10^6 PEM.

Studies of the inhibition of the reductase in leukocytes had mostly been performed with N-ethylmaleimide (NEM), a sulfhydryl antagonist. Not only did the presence of NEM inhibit the activity of the reductase but also the increased glucose oxidation and production of hydrogen peroxide following phagocytosis. As noted in Chapter V, other inorganic anions have been shown to inhibit the GSSG-R when it was studied as a purified protein. However, no studies

with these agents in a macrophage-containing system have been reported. The exposure of mice to 2.5 and 10 mg V/kg doses for 6 weeks resulted in significant GSSG-R activity decreases of 44% and 66% of in the PEM, respectively.

Not surprisingly, the degree of lowered PEM GSSG-R activity relative to the controls following vanadium treatment was similar to that observed with the G6PDH. This demonstrated the dependence of these two enzymes in the cell. However, in studies with commercially-prepared enzymes, the dehydrogenase appeared to be more sensitive than the reductase to the inhibitory effects of vanadate (Chapter V).

With decreases in activity of both G6PDH and GSSG-R, the production of superoxide anion following phagocytosis was expected to decrease. Based on the PMN work by Strauss et al. (1969), a decrease in GSSG-R activity (which normally increases as a consequence of membrane perturbation) would result in less NADPH (from secondarily enhanced G6PDH activity) for the function of NADPH oxidase. Consequently, cellular production of superoxide anion ($O_2^{\cdot}$) from vanadium-treated mice would decline compared to control cells.

The production of superoxide anion was shown here to decrease in a dose-dependent manner following vanadium treatment of the hosts for 6 weeks. The qualitative nitro-blue tetrazolium reduction study yielded a population of 41%-45% positive cells from control mice, a value previously observed with resident, non-elicited mouse PEM

(Johnston et al., 1978; Godfrey and Wilder, 1984). The 2.5V treated mice yields were 26% positive while those from the 10V mice were only 18%. Although quantitative studies of NADPH oxidase activity or assays of ferricytochrome c reduction would have better described the effect of vanadium treatment on superoxide production, the use of qualitative studies has been shown to reflect the status of macrophages exposed to exogenous agents or during diseased states of the host (Baehner and Nathan, 1968; Johnston et al., 1978; Catterall et al, 1986).

The present study was based upon the reduction of the quarternary nitrogen-containing indicator NBT consumed concurrently with the serum-opsonized zymosan. The possible contribution from the effects of vanadium on PEM phagocytosis had to be considered. In an earlier study, a 6-week treatment with 2.5V or 10V decreased PEM phagocytic activity by 13% and 21%, respectively, compared with the controls (Cohen et al., 1986). In the present study, the decrease in NBT-positive cells was 40% and 58%, respectively, and so the effects from decreased NADPH oxidase activity or that of the reductase/dehydrogenase system may have accounted for the remaining 27% and 37% (assuming the vanadate-induced effects were additive).

If the relative activities of GSSG-R for the 2.5V and 10V systems (56% and 34%) were used to predict the expected percentage of positive cells, the positive populations would be 24% for the 2.5V mice and 15% for the 10V mice (using 43%

as the control level). This decrease in superoxide production likely contributed to the previously observed increased lethality of viable Listeria in vanadium-treated mice. Superoxide anions have been shown to play a major role in the antilisterial activity of PEM (Nathan and Nakagawara, 1982; Ropitt, 1984; Hashimoto et al., 1986). The results of this superoxide study as they relate to PEM killing of Listeria were described in Chapter III.

Of the three enzymes studied here, it appeared that the glutathione peroxidase (GSHPX) has the most limited role in the processes of phagocytosis and oxygen metabolite production and/or destruction. This enzyme primarily functions to destroy cellular peroxides (in the form of hydrogen peroxide or lipid/organic peroxides) which result from peroxide leaching from the phagolysosomes.

Gee et al. (1971) reported an activity in rabbit PAM of 20 nmole NADPH oxidized/min per 10^6 PAM. Murray and co-workers (1980) reported a value of 60 nmole NADPH oxidized/min per mg protein using intact quiescent mouse PEM in culture. Gee et al. (1970) had earlier described the effects of homogenization versus freeze-thawing to release cytoplasmic enzymes. They noted that nearly all the peroxidase activity remained in the freeze-thaw supernatant while in homogenates the activity remained in the particulate fraction. By direct comparison, the activity was found to be 2.5 times greater in the freeze-thaw supernatant than in a homogenate. The work by Murray et al. (1980) employed

a homogenate of cultured PEM, and accounting for these experimental factors, would have yielded a GSSG-R activity of 1200 nmole NADPH oxidized/min per mg lysate protein. In this study, GSHPX activity in control mice ranged from 982-1072 nmole NADPH/min per mg protein.

Unlike the G6PDH and GSSG-R, the effects of exposure of mice to vanadate for 6 weeks did not cause a significant decrease in GSHPX activity at the lower vanadium dose. The activity in the 2.5 mg V/kg mice was only significantly lower than that obtained from phosphate buffer-treated mice ($P < 0.05$). The higher vanadium dosage (10V) still resulted in significant depressions of enzyme activity. The decrease in GSHPX activity relative to the controls was only 10% for the 2.5V mice and 47% for those that received 10 mg V/kg.

It was expected that the activity of GSHPX would mirror that of GSSG-R, since the peroxidase is dependent upon the latter for the regeneration of reduced glutathione substrate. What was observed were activities 20%-30% greater (relative to the controls) than the reductase activities at corresponding vanadium doses. This suggested that GSHPX activity may be maintained in the PEM but at the cost of cellular GSH. Unlike GSSG-R which appeared to have its activity adjusted to correspond with that of the G6PDH, the GSHPX may have superceded the decrease in reductase activity in order to protect the PEM against any buildup of peroxide. Macrophage viability remained unaffected during the 6 weeks of host pretreatment. The level of PEM GSH would then be

expected to be lower in vanadium-treated mice than in the controls and, conversely, GSSG levels would be greater.

The levels of total GSH (GSH + GSSG) encountered in the control PEM (36 nmole/mg protein) were consistent with the value reported in mouse PEM (31 $\pm$ 4 nmole/mg) by Rouzer et al. (1981). However, the level of GSSG was not reported for these cultured cells. In a study using rabbit PAM, Vogt et al. (1971) reported a level of 0.35 $\pm$ 0.11 nmole GSSG/10^6 PAM; the control values in this study using mouse PEM was 0.16-0.17 nmoles/10^6 PEM.

If the amount of protein expected in 10^6 PEM (0.08 mg: Cohn, 1978) were used to estimate what the level of free GSH might have been in intact cells as opposed to that in the cytolysate, a value of 2.86-3.74 nmoles would result; the level reported by Vogt in rabbit PAM was 5.9 nmoles GSH/10^6 PAM. It is expected that levels of GSH would be greater in PAM because they are in an oxygen-rich environment and encounter increased levels of active oxygen metabolites. Values obtained for free GSH in the present study may be low since it had been shown that unlike red blood cells, macrophages might lose free GSH by seepage into the supernatant media during washings (Vogt et al., 1971).

Several studies of the effect of alteration of the GSSG:GSH ratio upon glucose utilization, HMS activity, and phagocytosis have been performed. In erythrocytes, Scheuch and Rapoport (1960) showed that this ratio may determine the overall rate of glucose utilization. Jacob and Jandl (1966)

noted that it was the GSSG that ultimately controlled glucose utilization through induced alterations in HMS activity. Holmes-Gray et al. (1971) indicated that although decreased GSH levels in leukocytes did not interfere with phagocytosis, the rates of oxygen uptake, NBT reduction, and HMS activity were all diminished. Values for the ratio of GSSG:GSH in mouse PEM, either in culture or freshly harvested, have not been reported.

It was previously observed that there was no reduction in the viability of PEM from mice treated for 6 weeks with vanadium. The possible toxicity from a buildup of intracellular GSSG could be prevented by its transport out of the cell (Srivastava and Beutler, 1969; Vogt et al., 1971). Though GSSG levels in the PEM were increased by vanadium treatment, a toxic level was evidently not reached. This indicated that although enzymes involved in the GSH redox cycle were inhibited, the activity loss was not enough to completely block the ability to handle this potential toxin.

Peroxide levels were increased in tissues of animals or cell cultures exposed to vanadium (Inouye et al., 1980; Donaldson and LaBella, 1983; Donaldson et al., 1985); the relationship between vanadium treatment and reduced glutathione levels was not discussed in these reports. Interestingly, in the harvesting of PEM it was noted that the yields from the 10V mice were always low. A possibility of cell death and interference in recruitment due to vanadium effects upon DNA have been discussed earlier.

A second explanation for the unexpected pattern of GSHPX activity was that ingested peroxides from other leukocytes, as described earlier with the eosinophil MPO, may have contributed to the measured activity (Cohn and Wiener, 1963; Atwal, 1971). If, as proposed in this study, following vanadium exposure there was a decrease in PEM ability to degrade protein or intact microorganisms, then this may have allowed for engulfed peroxidase to persist in phagosomes. This could then enhance the assayed peroxidase activity of these PEM relative to what was expected based upon the activities of the other enzymes of the glutathione redox cycle.

These studies have clearly shown that IP exposure of mice to vanadium for 6 weeks could effect three cytoplasmic enzymes crucial to PEM function as well as to maintaining levels of substrate (GSH or GSSG) required for their proper activity. At both the level for aiding in energy production for phagocytosis and for the removal of activated oxygen metabolites, enzyme activities were depressed in a dose-dependent manner. This explained, in part, the earlier observed dose-dependent depression in PEM phagocytic capacities after vanadium exposure. The decrease in enzyme activities as well as the drop in production of superoxide anion may serve as the underlying basis for the enhanced susceptibility of these mice to viable Listeria.

CHAPTER V
IN VITRO VANADATE INHIBITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND GLUTATHIONE REDUCTASE

Introduction

Vanadium has been shown to inhibit several enzymes that utilize phosphorylated structures or inorganic phosphate as substrates. Those analyzed include alkaline and acid phosphatases (van Etten et al., 1974; Lopez et al., 1976; Seargent and Stinson, 1979), hexokinase, fructose-1,6-diphosphatase and 2,3-biphosphoglycerate synthase (Climent et al., 1981), phosphofructokinase (Choate and Mansour, 1978), DNA polymerase-alpha and terminal deoxynucleotidyl transferase (Sabbioni et al., 1983), ribonuclease (Lindquist et al., 1973), and glucose-6-phosphatase (Singh et al., 1981), to cite a few. The mechanism of inhibition in these systems has been ascribed to the ability of aqueous vanadate (VO_3^-) to assume a structural conformation similar to the active site-bound phosphate moiety (van Etten et al., 1974; Lopez et al., 1976; Chasteen, 1983). The structural similarity may allow vanadate to bind at the active site and (a) inhibit the binding of the proper substrate, (b) alter the enzyme secondary/tertiary structure, or (c) cause a decrease in the turnover time and subsequent regeneration of the active site.

The kinetic mechanism of G6PDH activity has been well studied in yeast (Levy, 1979; Alfolyan and Luzzatio, 1971), human erythrocytes (Soldin and Balinsky, 1968; Kosow, 1974), and pig and rat liver (Kanji et al., 1976a, 1976b). The mechanism involves an ordered sequential series of binding and release of substrates and products. The inhibition of yeast glucose-6-phosphate dehydrogenase (G6PDH) has been studied using metal halides such as mercuric and methyl mercuric chloride (Tsuzuki and Yamada, 1979), as well as with free phosphate, sulfate, and bicarbonate anions (Anderson and Nordlie, 1968; Anderson et al., 1968). In the latter studies, inhibition against the glucose-6-phosphate was competitive with inhibition constants of 9, 12, and 26 mM, respectively, and noncompetitive against the $NADP^+$ cofactor. However, it has also been reported that sulfate and phosphate were competitive inhibitors of $NADP^+$ binding but not against glucose-6-phosphate (Passonneau et al., 1966).

The major role of G6PDH in the cell is to provide reducing equivalents used (a) for enzymatic redox reactions, (b) to prevent oxidation of membrane unsaturated fatty acids, and (c) to optimize the activity of catalase for peroxide destruction in red blood cells. It is the first enzyme in a series whereby one molecule of glucose-6-phosphate eventually gives rise to a molecule of ribose-5-phosphate (for use in synthesis of ribo- and deoxyribonucleotides) and two molecules of NADPH. This enzyme cascade is known as the pentose phosphate pathway or hexose

monophosphate shunt. The non-enzymatic oxidation of NADPH to $NADP^+$ by vanadate has been shown in several systems (Ramasarma et al., 1981; Heide et al., 1983; Patole et al., 1986) as well as in the organs of animals dosed with vanadate (Bruech et al., 1984). However, the effects of vanadate on this enzyme yielding the first reducing equivalent has not been well studied. Gresser et al. (1984) and Nour-Eldeen et al. (1985) reported that vanadate appeared to indirectly stimulate the enzyme activity.

Glutathione reductase (GSSG-R) is a cytoplasmic enzyme which regenerates reduced glutathione (GSH) for use as a substrate for glutathione peroxidase. The latter enzyme is responsible for the destruction of cellular peroxides by their conversion to corresponding primary alcohols and water. Vanadate exposure has been shown to result in increased levels of peroxides in cultured cells (Inouye et al., 1980; Stacey and Klaassen, 1981a; Donaldson and LaBella, 1983) as well as in the intact organs of vanadate-exposed animals (Donaldson et al., 1985). Glutathione has been suggested to serve as the major reducing agent for the conversion of intracellular vanadate to vanadyl (Macara et al., 1980; Heide et al., 1983; Degrum, 1986). Vanadate treatment has also been shown to reduce the level of GSH in organs and in cultured cells (Stacey and Klaassen, 1981b; Bruech et al., 1984; Younes et al., 1984; Bracken and Sharma, 1985). The oxidation and conversion of GSH to a vanadyl-thioester has been suggested as the means of

cellular GSH depletion (Legrum, 1986). Complexation of vanadate with GSH has been documented (Macara et al., 1980; Nechay et al., 1986), but similar studies using the oxidized glutathione substrate (GSSG) of GSSG-R have not been reported.

Glucose-6-Phosphate Dehydrogenase Study

Materials and methods

Chemicals

Ammonium metavanadate (NH_4VO_3, 99.9% pure, J.T. Baker Chem. Co., Phillipsburg, NJ) was prepared as a 22 mM solution in Tris buffer (8 mM, pH 8.0). The metal was dissolved by heating over a low-heat source for 5 min and then filtered to remove contaminants. The final pH was readjusted to 8.0 by dropwise addition of 1% $NaHCO_3$ (w/v). The vanadate was prepared at this concentration so that the maximum strength of 4 mM could be delivered in a 0.55 ml aliquot. This aliquot was added to the reaction mixture to yield a final reaction volume of 3 ml.

The monosodium salts of glucose-6-phosphate and $NADP^+$ were purchased from Sigma Chemical Co. (St. Louis, MO). Glucose-6-phosphate (G-6-P) was prepared as a 7.5 mM aqueous stock solution and $NADP^+$ as a 1.5 mM stock solution in 1% $NaHCO_3$. Each stock solution was appropriately diluted in order to obtain the final working strengths of substrates needed for construction of the Lineweaver-Burk and Dixon plots used in determining inhibition kinetics parameters.

Yeast glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49, Type IX from baker's yeast [Saccharomyces cerevisiae]) was obtained from Sigma Chemical Co. The enzyme purity was approximately 80% (w/w) and had a specific activity of 260 units/mg protein; one unit was defined as 1 micromole $NADP^+$ reduced per minute at 25°C. The enzyme was dissolved in ice-cold Tris buffer to yield a solution of 1 unit/ml Tris. The buffer at pH 8 allowed for maximum activity to be utilized during all experiments (Avigad, 1966).

Ammonium chloride (NH_4Cl) was prepared as a 22 mM aqueous solution and served as a control to monitor the possible contribution to inhibition by the ammonium ion. A phosphate solution was prepared as a 0.33 M stock solution in Tris buffer with subsequent dilution to yield the final strengths needed for the Theorell-Yonetani analysis; vanadate was prepared as a 33 mM solution as described above.

NADPH (Sigma) solution was prepared as a 4.2 mM aqueous stock for the fluorescence studies and as a 10 mM solution in D_2O (Merke Sharpe and Dohme Canada Ltd., Montreal) for the NMR study.

Rate measurement

The measurement of the enzymatic reaction rate, the conversion of $NADP^+$ and G-6-P to NADPH and 6-phosphogluconate, respectively, was performed at room temperature in a 1 cm quartz cuvette in a Perkin-Elmer double beam spectrophotometer (Model Lambda 3). The rate was monitored and defined as the change in absorbance at 340 nm per minute.

The absorbance was recorded every 5 sec for a period of one min after initiation of the reaction.

The reaction system was composed of 2.10 ml of doubly-distilled water, 0.10 ml of aqueous 0.3 M $MgCl_2$, 0.10 ml of $NADP^+$ solution, 0.10 ml of G-6-P solution, and 0.55 ml of Tris buffer or the Tris buffer-vanadate solution. The volume of the last component was fixed at 0.55 ml but the ratio of the Tris and Tris-VO_3 volumes was varied in proportion to the amount of vanadate desired. In all cases, the vanadate was added 15 sec prior to the addition of the enzyme. The reaction was initiated by the addition of 0.05 units of G6PDH (1 U/ml solution). After 15 sec to allow for equilibration, the values were recorded for one minute. Tris buffer served as the absorbance blank.

In both the control and vanadate-containing assays, the G-6-P final concentrations were varied from 0.10-0.25 mM and the $NADP^+$ from 0.025-0.050 mM. For each substrate concentration, all levels of the second substrate were analyzed both with and without the presence of vanadate. Each substrate level was analyzed five times.

From the change at 340 nm in absorbance per minute, the slope was calculated and this served as the "activity" for the run. The activity is actually a mathematical modification of this slope (B_1) using the value of the extinction coefficient for NADPH (at 25°C, 6.317 x 10^3 M^{-1} cm^{-1}); for the purpose of this study, the conversion to "activity" would alter neither the magnitude nor the pattern of

inhibition observed. Using the slope value, B_1, Lineweaver-Burk plots ($1/B_1$ vs $1/[S]$) and Dixon plots ($1/B_1$ vs $[I]$) were constructed and the patterns of inhibition and inhibition constants (K_i) for both substrates were calculated.

To determine if ammonium ions contributed to inhibition, the activity of the enzyme was measured in the presence of equivalent amounts of ammonium ion from different sources; either ammonium chloride or ammonium metavanadate. The rates were determined using the reaction system described above. The specific activity was calculated as units of activity per mg enzyme present in the system.

Phosphate inhibition of the G6PDH has been well documented, and so the possibility of vanadate using a similar inhibitory mechanism was evaluated. Theorell-Yonetani analysis was employed to determine if the two inhibitors bound the G6PDH in a mutually exclusive manner (Yonetani and Theorell, 1964). Phosphate in Tris buffer was added along with vanadate 15 sec prior to the addition of the enzyme. The phosphate and vanadate were prepared as 0.33 and 0.033 mM solutions, respectively, and diluted for use in the assays. All rate measurements were performed as above.

Oxidation of NADPH by vanadate was monitored after mixing NADPH (0.04 mM) with several levels of vanadate (0.33-4.00 mM) in the presence and absence of enzyme. In these experiments, no G-6-P or magnesium cofactor was added. The decrease in absorbance at 340 nm was recorded every 30 sec for a period of 15 min.

Effect of vanadate on NADPH fluorescence

The effect of vanadate on the fluorescence of NADPH was analyzed using a Perkin-Elmer fluorescence spectrophotometer (Model LS-5). The NADPH (0.04 and 0.14 mM) was mixed with vanadate at final concentrations of 0.11-1.10 mM in a 3 ml reaction vessel. The remaining component in the system was the Tris buffer only. The excitation wavelength was set at 340 nm with a slit width of 5 nm. The fluorescence emission was recorded at 450 nm.

In order to determine the association constant of vanadate with the NADPH/$NADP^+$, a fluorescence quenching titration was performed. Using a level of 0.5 mM NADPH, vanadate was added to yield final concentrations of 0.58-7.00 mM in the 3 ml system. The fluorescence intensity was monitored over the range of 300-520 nm. From the degree of fluorescence quenching in the presence of varied levels of vanadate, the association constant (K_a) was calculated using the equation of Attallah and Lata (1968). The ratio of metal:NADPH used was on the same order as that used in the enzyme inhibition studies.

NMR analysis of vanadate:NADPH complexation

The NMR spectra were determined at 300 MHz in a Nicolet NT-300 wide-bore NMR spectrometer in the laboratory of Dr. W. Brey, at the Department of Chemistry at the University of Florida. All assays were run in a 5 mm sample tube using D_2O as the solvent and the reference. Vanadate, NADPH, and the NADPH:vanadate mixture were prepared in D_2O as described

earlier, and no Tris buffer was included. The final concentration of NADPH and vanadate was 5mM and 20 mM, respectively. All chemical shifts were referenced to the D_2O proton signal (4.6 ppm). Each magnetic cycle lasted 10 sec.

Results

The inhibition by vanadate against the yeast G6PDH in the Tris buffer system was competitive against the $NADP^+$ cofactor and mixed-type against the G-6-P (Figure 23) as indicated by their respective double reciprocal plots. Using Dixon plots of 1/activity versus inhibitor concentration or the slope values from the Lineweaver-Burk plots, the inhibition constants for each substrate were determined. The K_i value was 2.7 mM with $NADP^+$ and 2.1 mM with the sugar phosphate. The level of bicarbonate ions in the system (from the final pH adjustment step in vanadate preparation) were two orders of magnitude below the reported K_i and were not inhibitory when tested in a separate study. In all rate experiments, the concentration ratio of the inhibitor to substrate was never greater than 40:1. When the ratio was raised to 200:1, similar inhibition patterns resulted.

When two inhibitors were present in the system (phosphate and vanadate), Theorell-Yonetani analysis indicated that each was a mututally exclusive inhibitor in this system. This was apparent from the resulting parallel lines

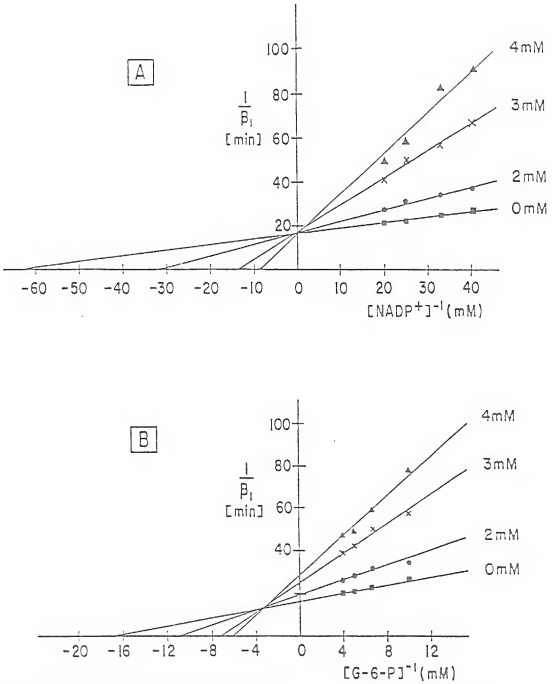


Figure 23. Lineweaver-Burk analysis of the inhibition of glucose-6-phosphate dehydrogenase by vanadate. Ammonium metavanadate with (A) $NADP^+$, or (B) glucose-6-phosphate (G-6-P) as the varied substrate in a 3 ml volume at 25°C. In (A), glucose-6-phosphate concentration was 0.150 mM, and $NADP^+$ varied from 0.025-0.050 mM. In (B), $NADP^+$ concentration was 0.04 mM, and G-6-P varied from 0.10-0.25 mM. Enzyme (0.05 U) and Mg^{2+} (10 mM) concentrations were held constant. Vanadate (in Tris buffer: 8 mM, pH 8) levels ranged from 0 to 4 mM. B_1 was the change in absorbance at 340 nm/min.

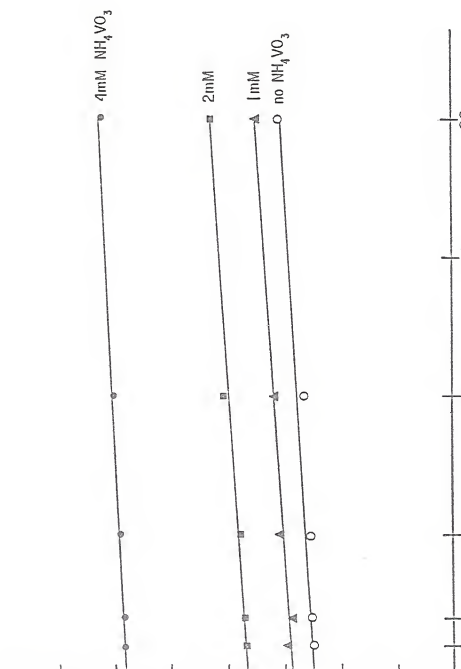
(Figure 24) when the enzyme activity was analyzed at fixed levels of one inhibitor (in this case, vanadate) while varying the levels of the second inhibitor (phosphate).

The value of 4 mM vanadate as the maximal level of inhibitor employed was based on observations of the effect of coincubation of vanadate with both $NADP^+$ and with NADPH. In both cases, a dose-dependent hyperchromic shift was observed at the absorbance maxima for the adenine moiety (260 nm), with hypochromicity observed at 340 nm with NADPH (Figure 25). No bathochromic or hypsochromic shifts were detected. In order to obtain absorbance values that were reliable (no greater than 2.00), the level of vanadate used was strictly controlled as was the maximal amount of $NADP^+$ which could be employed in the assays.

The effect of added magnesium was also assayed for possible complexation with the $NADP^+$ cofactor. The role of magnesium as a cofactor in this enzyme reaction was well established by Lee and Kosicki (1967), as well as its ability to ameliorate inhibition by inorganic phosphate and ATP. The absorbance patterns of $NADP^+$ and NADPH were not altered by the addition of Mg^{2+} in the form of aqueous $MgCl_2$ nor was Mg^{2+} effective in lessening the enzyme inhibition by added vanadate.

The possible effect of the ammonium ion behaving as an inhibitor was also examined. Using equivalent levels of ammonium ion (NH_4^+) from the metavanadate and from ammonium chloride, the minimal contribution to inhibition by this ion

Figure 24. Theorell-Yonetani analysis of the inhibition of G6PDH using vanadate and phosphate ions. Plot is enzyme rate vs phosphate concentration at varying concentrations of NH_4VO_3: no NH_4VO_3 (○), 1 mM (△), 2 mM (▨) and 4 mM (◍). G-6-P (0.15 mM), $NADP^+$ (0.04 mM), Mg^{2+} (10 mM) and enzyme (0.05 U) were present in the final reaction volume of 3 ml maintained at 25°C. B_1 was described in Figure 23.


4mM NH_4VO_3
2mM
1mM
no NH_4VO_3
$\frac{1}{P_i}$ [min]
70
60
50
40
30
20
10
1
2
5
10
15
20
Phosphate Concentration (μM)

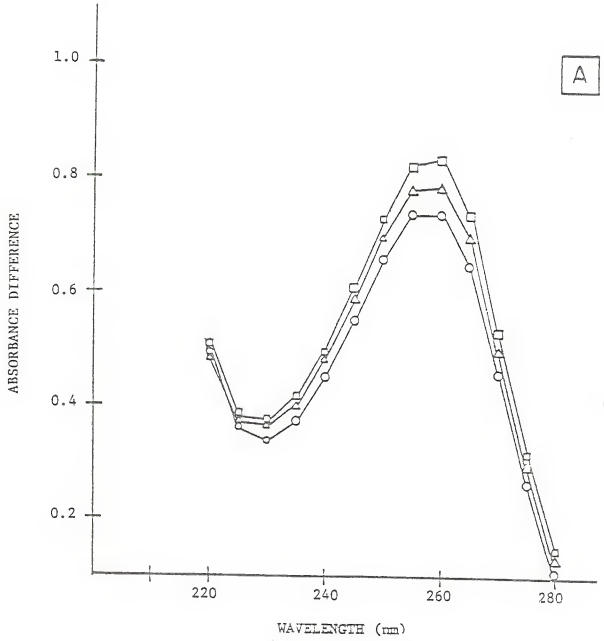


Figure 25. Absorbance spectra of $NADP^+$ and NADPH in the presence and absence of vanadate. Absorbance difference spectra of the mixture of (A) $NADP^+$ and (B) NADPH with vanadate in Tris buffer (8.0 mM, pH 8) at 25°C in a final volume of 3 ml. (○) 0.04 mM cofactor only plus 0.33 mM (□) or 0.55 mM (△) vanadate.

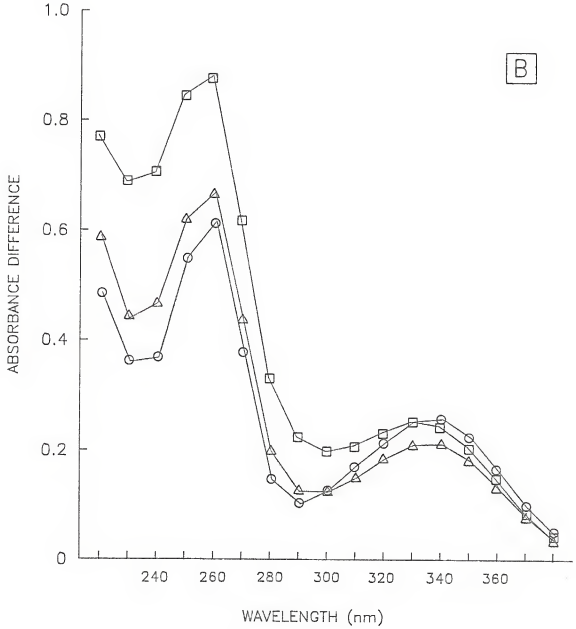


Figure 25--continued

can be observed in Figure 26. At levels of NH_4^+ up to 0.02 mmole, the ammonium chloride-containing system displayed a loss of only 8.3% in the calculated specific activity. However, from an equivalent level of ammonium ion as found in NH_4VO_3, the loss was almost 50%. This demonstrated that the ammonium ion had little effect on enzyme activity and that any observed inhibition was due primarily to the vanadate ions.

As mentioned above, the mixing of vanadate with $NADP^+$ and with NADPH led to an alteration in the absorbance amplitude at the respective wavelength maxima. However, because of the known oxidative role of vanadate, the possibility of oxidation of the reaction product (NADPH) back to substrate was assayed. Incubation of NADPH with vanadate over a period of 15 min resulted in a continuous decrease in the 340 nm absorbance. The rate of oxidation was only dependent on the initial concentration of vanadate present. The rate varied from 48 pmole/min in the presence of 0.77 mM vanadate, to 750 pmole/min with 4 mM vanadate. The addition of enzyme to the mixture had no effect on the rates of oxidation. The oxidative rate was calculated to be no more than 5% of the forward reaction rate observed in the reaction showing the maximal inhibition by vanadate. Therefore, vanadate was actually a stronger inhibitor than calculated since the forward rate was still below that of vanadate-free controls that accumulated inhibitory NADPH.

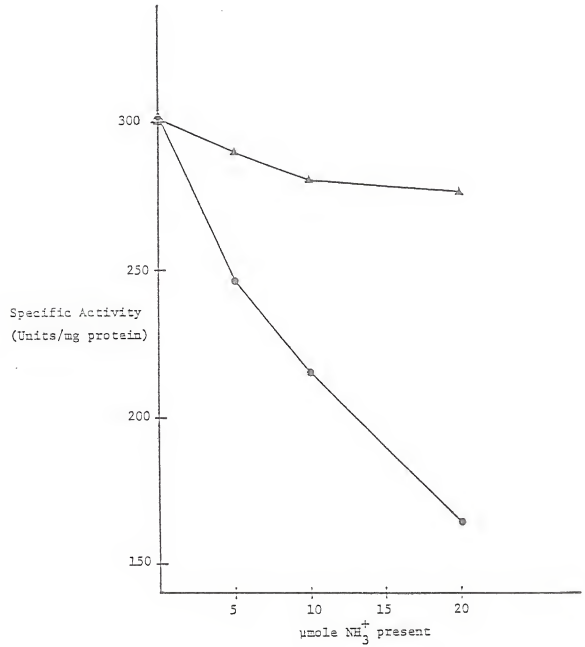


Figure 26. Effect of ammonium ion sources on the activity of yeast glucose-6-phosphate dehydrogenase. Enzyme activity measured in the presence of $NADP^+$ (0.02 mM), G-6-P (0.1 mM), Mg^{2+} (10 mM) and enzyme (0.05 U). Ammonium ion was initially 22 mM as NH_4Cl (▲) or NH_4VO_3 (●). Equal volumes of each were analyzed separately.

When a solution containing only NADPH (0.14 mM) was mixed with increasing levels of vanadate, a quenching of fluorescence at 450 nm was observed (Figure 27). An appreciable drop in the intensity was not apparent until levels of VO_3 were greater than 0.33 mM. When the assay was repeated using a lower level of NADPH (0.04 mM), a similar quenching pattern resulted. This indicated that vanadate:NADPH complexes were formed quantitatively regardless of the concentration of cofactor. This information was useful in the interpretation of the enzymatic rates since this indicated that the apparent extinction coefficient of NADPH was unaffected by the metal.

Further evidence of the interaction of vanadate with NADPH at a site other than the pyridine moiety was provided by proton NMR analysis. Using 300 MHz pulses (Figure 28), the transverse relaxation time ($1/T_2$) of the dihydropyridine moiety of NADPH (PC_4H_2) at 2.50 ppm was not altered nor was there any shift in the signal. Upon complexation with vanadate, there was an increase in the integrated intensity of the pyridine C_2H peak at 6.70 ppm. Other new signals in this region were indicative of the presence of oxidized $NADP^+$ in this mixture.

In the region of the adenine moiety (8.00 ppm), when vanadate was present there was an increase in the integrated intensities of the adenine AC_8H and AC_2H signals. There was also the appearance of several highly resolved new signals in the region. These peaks were not present in the 8.50 to

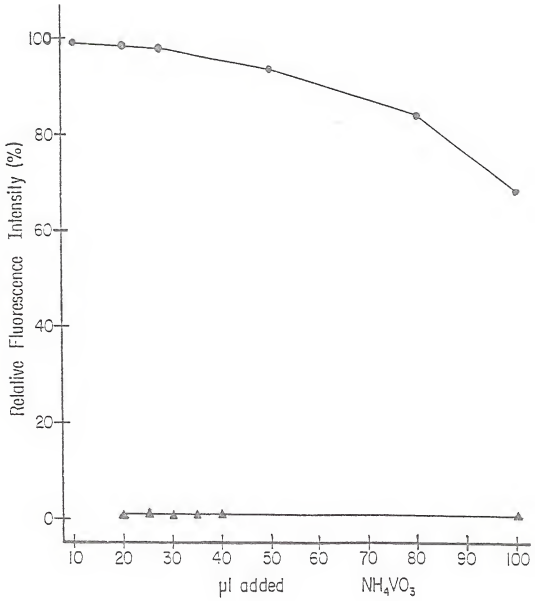


Figure 27. Preliminary analysis of the quenching of NADPH fluorescence in the presence of vanadate. Relative fluorescence intensity of NADPH (0.14 mM) with vanadate (●) or of metal only (▲), with excitation wavelength of 340 nm, emission wavelength of 450 nm and slit width of 5 nm. Initial vanadate concentration = 33 mM.

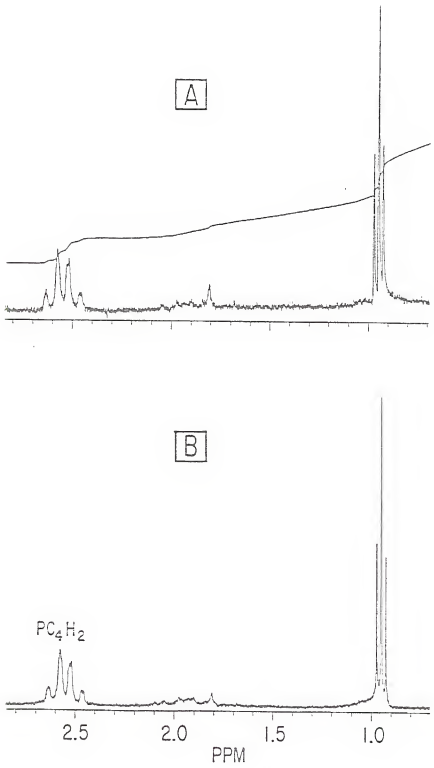


Figure 28. Proton NMR analysis of NADPH in the presence and absence of vanadate ions. Spectra of (A) NADPH (5 mM) in the presence of vanadate (20 mM) in D_2O and (B) NADPH (5 mM) only. Spectra determined at 300 MHz using 5 mm sample tube with D_2O as the internal standard (4.6 ppm).

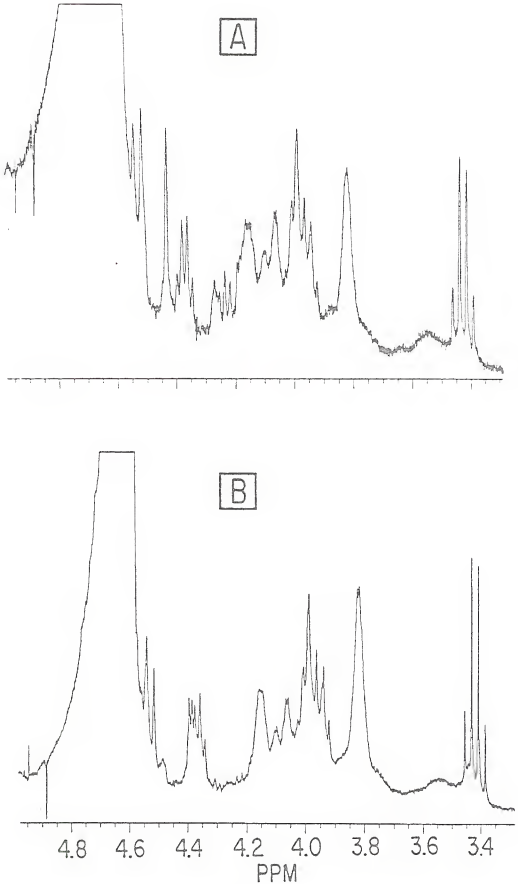


Figure 29--continued

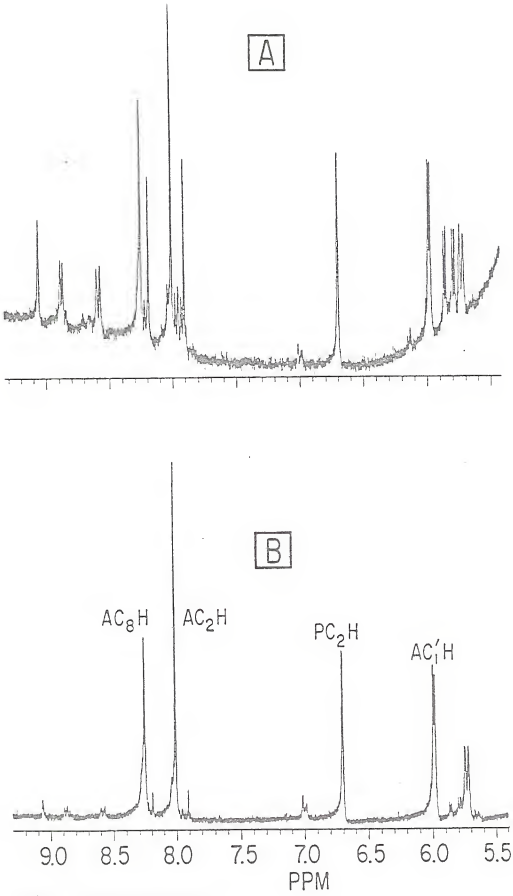


Figure 29--continued

9.00 ppm region of the native NADPH spectrum. Signal enhancement in the 3.60-4.50 ppm range also occurred when vanadate was added; a very sharp signal at 4.65 ppm and a new triplet at 4.45 ppm appeared. The reference peak for D_2O was set at 4.60 ppm.

To determine the strength of this association between vanadate and the NADPH, fluorescence quenching titration was performed. Using 0.5 mM NADPH, and adding increasing levels of vanadate (0.58-7.00 mM) the decrease in fluorescence was monitored over the 300-520 nm range. As shown in Figure 29, there was a dose-dependent decrease in the fluorescence intensity with increasing vanadate levels. The association constant was calculated from the drop in intensity using the equation of Attallah and Lata (1968) whereby:

$$K_a = \frac{Q_f}{(1-Q_f)(D_t - nQ_f)C}$$

with C = concentration of NADPH, Q_f = the quenching fraction, D_t = concentration of vanadate present, and n = the metal:cofactor concentration ratio. The association constant (K_a) for vanadate was calculated to be $4.0 \times 10^2\ M^{-1}$ at 25°C and pH 8.0.

Discussion

This study was undertaken to determine the interaction between vanadate and glucose-6-phosphate dehydrogenase. The

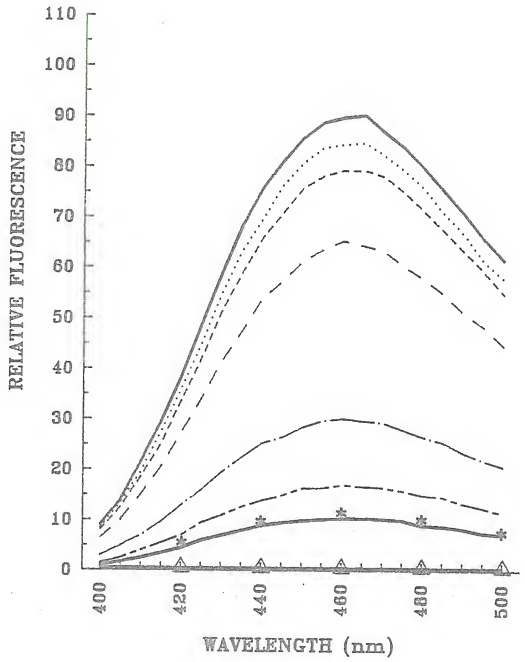


Figure 29. Quenching of NADPH fluorescence in the presence of vanadate ions. NADPH (0.5 mM) was combined with vanadate in a final reaction volume of 3 ml. Levels of vanadate were 0, 0.58, 1.16, 2.33, 4.66, 5.83, and 7.00 mM (lines from top to bottom, respectively). The line along the x-axis was the fluorescence of the metal alone. Slit width was 5 nm, excitation wavelength was 340 nm and emission wavelength was 450 nm.

interaction of vanadate with the enzyme, its substrates and/or products would then be useful for explaining the changes in enzyme activity observed in peritoneal macrophages obtained from vanadium-treated mice (Chapter IV). The inhibition observed in this study provides a basis for developing a mechanism for the immunomodulation that was observed in earlier studies using mice (Cohen et al., 1986).

The inhibition of the yeast G6PDH by vanadate ions was on the same order of potency as observed with other inorganic anions such as phosphate or sulfate (Anderson et al., 1968). However, the patterns of inhibition placed vanadate in a class by itself. Both the phosphate and sulfate displayed competitive inhibition with respect to the G-6-P substrate when an anion:substrate ratio of 200:1 or greater was studied. As this ratio was lowered, the pattern shifted to mixed-type. The K_i values for sulfate and phosphate were 12 and 9 mM, respectively, in a system maintained at pH 8. The inhibition by these two anions regarding the $NADP^+$ cofactor was noncompetitive. Vanadate displayed mixed-type inhibition against G-6-P regardless of the inhibitor to substrate ratio. At ratios of 40:1 and 200:1, the pattern remained mixed-type. The inhibition with respect to the $NADP^+$ was always competitive. The K_i values calculated for the G-6-P and the $NADP^+$ were 2.1 and 2.7 mM, respectively.

The Theorell-Yonetani analysis using a dual inhibitor system indicated that vanadate and phosphate were mutually

exclusive inhibitors against the yeast enzyme. This would mean that the two anions shared a common binding site on the G6PDH. However, different patterns of inhibition were observed with respect to both substrates for these two inhibitors as noted above.

This enzymatic reaction followed an ordered sequential mechanism whereby the $NADP^+$ was bound first and the product NADPH was released last (Kosow, 1974; Kanji et al., 1976a, 1976b). Koshland (1964) postulated that the enzyme active site was flexible and that binding of the first substrate ($NADP^+$) induced a change in the conformation of the active site region which then yielded a more favorable form for binding the second substrate (G-6-P). Anderson et al. (1968) noted that the noncompetitive nature of sulfate and phosphate regarding $NADP^+$ would mean there would be no blockage of the first binding step. These two anions could, however, mimic the phosphate moiety of the G-6-P and so bind to the active site after the $NADP^+$ was bound. This would induce a "flexation" of the catalytic region and inhibit the binding of G-6-P. Although vanadate and phosphate were mutually exclusive inhibitors, this mechanism appeared not to apply to vanadate since the pattern of inhibition was not the same as for phosphate.

In solution, $NADP^+$ and NADPH have inter- as well as intramolecular interactions of their heterocyclic rings. Generally, beta-$NADP^+$ and beta-NADPH have the adenine and pyridine rings stacked in parallel (Heyn and Brets, 1975).

In this state, there occurred intramolecular charge-transfer interactions with the adenine ring acting as the electron donor and the quarternary pyridine as the acceptor. The direct transfer of energy from the purine to pyridine moiety gave rise to the fluorescence spectra of $NADP^+$ when 260 nm light was the excitation source (Weber, 1958; Cilento and Shreier, 1964; Shifrin, 1964). It was also shown that the amino group of the adenine was necessary for the proper intramolecular transfer of energy (Shifrin and Kaplan, 1961). It was concluded that these charge-transfer interactions did not cause the observed base stacking, but it was just the opposite which ultimately gave rise to long wavelength absorbances for $NADP^+$ (Ross et al., 1982).

At low pH or with high levels of salt, unstacking of the adenine and pyridine occurred (Johnson and Schleich, 1974). The lower pH resulted in protonation of the adenine and so, electrostatic repulsion of the charged rings occurred. The salt presence introduced alterations in the electron environment around the non-binding electrons of the purine. In both cases, this led to a decrease in the associability of the rings and, ultimately, unstacking. With this unstacking, there was an observed hyperchromicity at the adenine absorbance maxima (260 nm) as well as the appearance of longer wavelength bands (although very weak). The reduction of the pyridine ring also reduced the stacking factor and this contributed to the enhanced absorbance of the NADPH pyridine ring at 340 nm (Cross and Fisher, 1969).

This was due to the less favorable interaction of rings when an electrophilic nitrogen in pyridine was no longer charged.

With this understanding of the complex intramolecular associations in the cofactor, the effects of vanadate on the absorbance, NMR, and fluorescence spectra could then serve as a basis for deriving a mechanism for the observed changes in G6PDH activity in the presence of vanadate.

The absorbance studies with $NADP^+$ and NADPH indicated that an interaction occurred between vanadate and these compounds. The native absorbance spectra of $NADP^+$ contained the adenine contribution at 260 nm and in NADPH the 260 nm band and the reduced pyridine region at 340 nm. In the presence of vanadate, no hypsochromic or bathochromic shifts were detected for both forms, only concentration-dependent hyperchromicity was observed at the adenine moiety. As the concentration of vanadate was increased in the presence of $NADP^+$, a weak increase in absorbance at 340 nm was observed and this new band also displayed concentration-dependent hyperchromicity. However, from an analysis of the separate contributions to absorbance from the metal and the cofactor, no true enhancement of absorbance at the pyridine moiety was detected. The oxidation of NADPH by vanadate resulted in hypochromic changes at 340 nm, so a precise interpretation of metal-cofactor interactions at this wavelength was difficult. The structure of vanadate contained two carbonyl-like groups (vanadyl) and a vanadium oxide

($V{-}O^-$) moiety, which exist in equilibrium. Studies with carbonyl compounds mixed with $NADP^+$ indicated addition reactions at the pyridine ring (Meyerhof et al., 1938; San Pietro, 1955) that gave rise to spectra identical to NADPH. However, these early studies did not mention hyperchomic shifts in the adenine region with carbonyl agents present.

The interaction of vanadate with the adenine, but not with the pyridine moiety, was confirmed by the proton NMR study. In the presence of vanadate, the C-4 protons (PC_4H_2, 2.59 ppm) did not undergo any chemical shift nor any alteration of the transverse relaxation time ($1/T_2$). However, NADPH still displayed hyperchromicities at 260 nm in the presence of vanadate. The NMR profile of the vanadate:NADPH mixture indicated little interaction at the pyridine ring; interaction was most prevalent with the adenine moiety.

Pentavalent vanadium is very electrophilic and so the electron-rich rings and the amine group of adenine provide the best sites for electrostatic interaction. This complexation of vanadate with the adenine rings would then lead to an unstacking in the manner similar to the high-salt experiments described earlier. By disturbing the environment of the non-binding electrons of adenine, interaction with the pyridine ring was decreased. The subsequent unstacking resulted in the hyperchromicities at the absorbance maxima for adenine in both $NADP^+$ and NADPH.

Complexation of the adenine ring had been correlated with a decreased fluorescence of NADPH at 450 nm when a 260

nm excitation source was used (Warburg and Christian, 1936; Shifrin and Kaplan, 1959), but had no effect when the source was 340 nm. Addition reactions of bases could occur with the reduced coenzyme, and this proceeded through an anion attack at the C-2 position on the pyridine ring (Anderson and Berkelhammer, 1958). The NMR analysis of vanadate with NADPH indicated that an interaction did occur at this C-2 site (PC_2H, 6.70 ppm) with a resulting enhancement of the proton signal. These addition reactions resulted in hypsochromic shifts of the pyridine band from 340 nm to 290 nm (Haas, 1937).

In this absorbance study, enhancement of the adenine 260 nm peak covered this shift and led to the conclusion that no hypsochromic shift of the 340 nm peak had occurred. Clearly, this could only be an interpretation and could not be conclusive on the basis of this study. The result of the shift would lead to decreased fluorescence of the NADPH:vanadate mixtures when only a 340 nm excitation was used and this was what was observed. From the evidence provided by the NMR, fluorescence, and absorbance studies, it has been shown that vanadate interacted directly with the adenine and possibly with the pyridine moiety (C-2 site). These interactions were confirmed by the observed alterations in the spectra ascribed to other anions and electrophilic species.

On the basis of these studies, the association constant (the measure of the strength of the complex formation) was

calculated to be 4.0×10^2 M^{-1}. In comparison, other metal complexes with $NADP^+$ and/or with NADPH displayed a wide range of strengths of binding. Mercuric chloride showed a strong complexation with K_a values of 1.0×10^4 and 3.9×10^4 M^{-1} for the $NADP^+$ and NADPH, respectively (Tsuzuki et al., 1979), while phosphate mixtures with oxidized cofactor yielded a K_a of only 0.52 M^{-1} (Ungar et al., 1979). This trend of strengths was mirrored in a comparison of the K_i values for these compounds with respect to the cofactor in the yeast G6PDH reaction. Phosphate was noncompetitive, vanadate (intermediate K_a) had a K_i of 2.7 mM, and mercuric chloride had a K_i of 0.022 mM (Tsuzuki and Yamada, 1979). The binding of $NADP^+$ most likely served as a basis for the observed patterns of inhibition with the yeast enzyme, since the vanadate patterns were more like that of mercuric chloride than of sulfate or phosphate.

Preliminary proton NMR studies of the binding of pyridine coenzymes to active sites suggested that it was either the adenine (Hollis, 1967) or the pyridine moiety (Jardetzky et al., 1963) which served as the binding region rather than the ribose rings or diphosphate linkages. Sarma and Kaplan (1970) concluded that the purine interacted first and that this subsequently facilitated the binding of the pyridine ring. This study indicated that vanadate interacted primarily with the adenine rings, and most likely this was enough of a steric alteration so as to prevent the cofactor from binding to the active site on the G6PDH. Any

structural modification of the active site or the $NADP^+$ cofactor was shown to cause enzyme inactivation by preventing the formation of an active enzyme tetramer (Yoshida, 1966; Robbins et al., 1975). This modification eventually led to the dissociation of the yeast enzyme to its nonfunctional monomeric units (Yoshida et al., 1967; Bonsignore et al., 1968).

That vanadate displayed competitive inhibition with respect to $NADP^+$ and mixed-type inhibition with G-6-P was confounding. The basis for competitive inhibition implied that the vanadate bound the enzyme and thus blocked the $NADP^+$ from the active site (i.e. both were mutually exclusive). However, this type of pattern would also arise when non-metabolizable analogs of the substrate were present. This study showed that vanadate formed complexes with the cofactor, and so this complex might compete with $NADP^+$ to bind the G6PDH active site. Because of the proposed change in the adenine/pyridine interactions, this also may have resulted in a bound coenzyme with a reduced ability to act as a hydrogen acceptor in the dehydrogenase reaction.

Interestingly, the patterns of inhibition using vanadate were similar to those observed with mercuric compounds (Tsuzuki and Yamada, 1979). The mercuric complexes that did not form strong complexes with the cofactor had no effect on $NADP^+$ binding with the yeast enzyme and were competitive inhibitors only against the G-6-P. Those that bound $NADP^+$ tightly displayed noncompetitive inhibition against G-6-P

substrate even at low levels of inhibitor. Vanadate, with a much stronger complexation of $NADP^+$ as compared with phosphate, displayed mixed-type inhibition (a form of noncompetitive inhibition) against G-6-P.

The Theorell-Yonetani analysis predicted that vanadate bound the enzyme in the same manner as phosphate. This would mean that, at the least, competitive inhibition should be observed with respect to the G-6-P at high levels of vanadate with a conversion to mixed-type at lower levels. What was observed was mixed-type inhibition at all levels of vanadate and G-6-P tested. The inhibition of the binding of $NADP^+$ was apparently of such great consequence that the effect of vanadate on G-6-P binding were secondary in the overall mechanism of inhibition.

The G6PDH reaction followed an ordered sequential mechanism and as Koshland postulated (1964), the binding of the $NADP^+$ was crucial for the alignment of the active site for acceptance of the G-6-P substrate. As with mercuric agents that formed complexes with the coenzyme, the vanadate ion was a stronger inhibitor of the yeast G6PDH than were other inorganic anions that did not form complexes. It was clear that the effect of vanadate on the first step of the enzymatic reaction led to the overall inhibition of activity of this yeast enzyme.

Glutathione Reductase Study

Material and methods

Chemicals

Oxidized glutathione (GSSG) and glutathione reductase (GSSG-R) from bovine mucosa were purchased from Sigma. The GSSG was prepared as a 60 mM aqueous solution in double-deionized water and NADPH was similarly prepared as a 10 mM solution. The GSSG-R obtained had a purity of 75% and a specific activity of 100 units/mg protein; one unit is defined as 1 micromole GSSG reduced per minute at 25°C at pH 7.6. The enzyme was dissolved in ice-cold 10 mM phosphate buffer (pH 7.6) to yield a solution of 0.5 U/ml.

Because of the previously observed effects on NADPH absorbance by the presence of vanadate, the enzymatic rate was calculated based on the formation of reduced glutathione (GSH) rather than the disappearance of NADPH. Ellman's reagent (DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid)) was prepared as an 8 mM solution in 5 mM phosphate buffer in order to monitor thiol formation. This level of DTNB was adequate to prevent the reoxidation of the GSH to GSSG by vanadate.

The vanadate was prepared as described earlier. The working concentration of the ammonium metavanadate was set at 0.23 M, with subsequent dilution yielding the concentrations of vanadate desired.

Rate measurement

The total ionic concentration of the reaction was maintained at 0.1 M in all the assays with or without the presence of vanadate. The reaction system was composed of 2.55 ml of phosphate or phosphate/vanadate solution, 0.10 ml of GSSG solution, 0.10 ml of water, 0.10 ml of the GSSG-R solution, and 0.10 ml of DTNB. After a 5 min preincubation at 25°C, the reaction was initiated by the addition of 0.05 ml of the NADPH solution. The increase in absorbance at 412 nm was monitored every 15 sec for a period of 3 min. A solution containing all reagents except the NADPH served as the absorbance blank.

The levels of vanadate were varied by mixing the vanadate stock solution with different levels of 0.23 M phosphate buffer. This allowed the final ionic concentration to be maintained at 0.1 M which represents the optimal concentration for anion activation in this enzyme system (Icen, 1967; Moroff and Brandt, 1973 and 1975).

Vanadate complexation with GSSG

Vanadate was prepared as an aqueous 0.23 M solution using doubly-deionized water and diluted to yield different concentration solutions. To 2.60 ml of the vanadate solution, 0.10 ml of the GSSG solution (at different concentrations) was added. Water was used to replace all the other components from the previous rate measurement assays. The mixture was subjected to a full spectroscopic scan

(210-600 nm) in a Perkin-Elmer double beam spectrophotometer The absorbance at 5 nm intervals was recorded and a composite curve was constructed. This was repeated three times for each level of vanadate/GSSG combination and a single average composite was prepared.

To determine the degree of complexation between the metal and GSSG, varied amounts of GSSG were added to a solution containing a fixed concentration of vanadate and the absorbance at 425 nm was recorded. From an analysis of the absorbance as a function of ligand (GSSG) present, the amounts of free and bound metal was calculated for use in Scatchard analysis.

Results

Unlike the yeast G6PDH, the pattern of inhibition of vanadate against the bovine mucosa glutathione reductase (GSSG-R) was not discernable due to the interaction of vanadate not only with the enzyme substrates, but with the products as well. Incubation of the GSSG-R with 0.1 M phosphate buffer led to maximum activation of the system. However, incubation with 0.1 M vanadate resulted in a complete loss of activity. If the level of vanadate was decreased and the total ionic concentration maintained at 0.1 M by supplementing with phosphate ions, activity was detected albeit depressed. As seen in Figure 30, the inhibition with increasing vanadate was dose-dependent, but did not appear to follow a 1:1 stoichiometry. From the

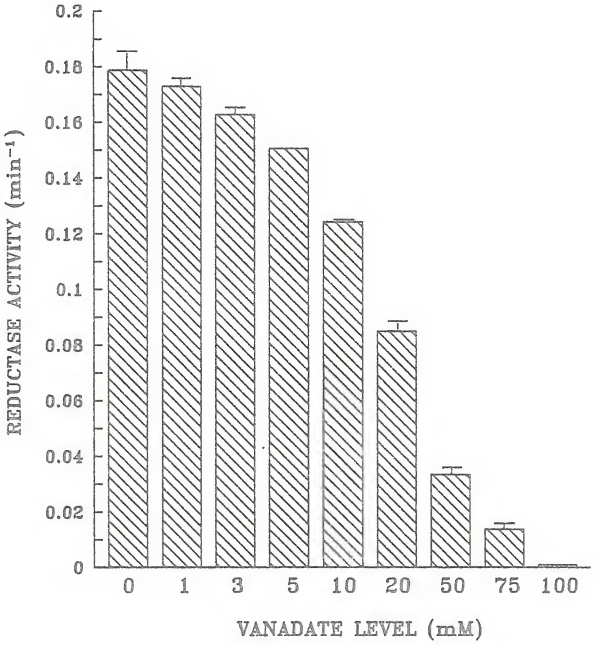


Figure 30. Glutathione reductase activity as a function of vanadate ion levels. Activity determined by the change in absorbance at 412 nm/min over a 3 min period. Final reaction was volume 3 ml. Bars equal to the mean (± SD) of 4 runs at each vanadate level. Total anionic concentration was maintained at 0.1 M (with phosphate ions) for all assays.

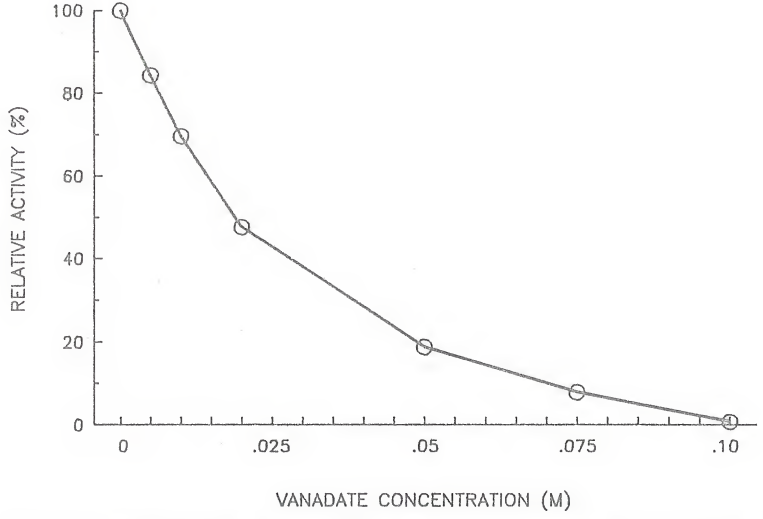


Figure 31. Relative activity of glutathione reductase as a function of the vanadate concentration. All points were activities compared to a vanadate-free control. Assay conditions were described in Figure 30.

degree of enzyme activity loss at each vanadate level, the IC_{50} was calculated (Figure 31). The 50% inhibitory concentration in this system was 19 mM vanadate.

When a fixed level of NADPH and varied levels of oxidized glutathione (GSSG) were tested in the absence of vanadate, a standard substrate:activity response was observed. However, when vanadate was added to the system, the double reciprocal plots needed for analysis of inhibition were highly irregular. These plots all showed that at very high levels of vanadate, the $1/B_1$ vs $1/[S]$ lines became flat over the entire range of substrates. These plots also showed a convergence in the (+X, +Y) region of the lines obtained with differing levels of inhibitor (Figure 32). All of these problems in plotting demonstrated the anomalous behavior of this enzyme in the presence of anions.

High levels of GSSG also caused inhibition as reported elsewhere (Massey and Williams, 1965; Staal and Veeger, 1969; Moroff and Brandt, 1975). The inhibition of activity in the present study was both a factor of the amount of GSSG and vanadate (Figure 33), with the degree of activity loss more dependent upon the level of the metal. When no Ellman's reagent was present to protect the reduced product, GSH, there was reoxidation by vanadate to the substrate GSSG. Although this allowed for a continuous repletion of substrate, the enzyme activity was still below that of the controls without vanadate. In a study using GSH as the substrate, where no activity was expected in the absence of

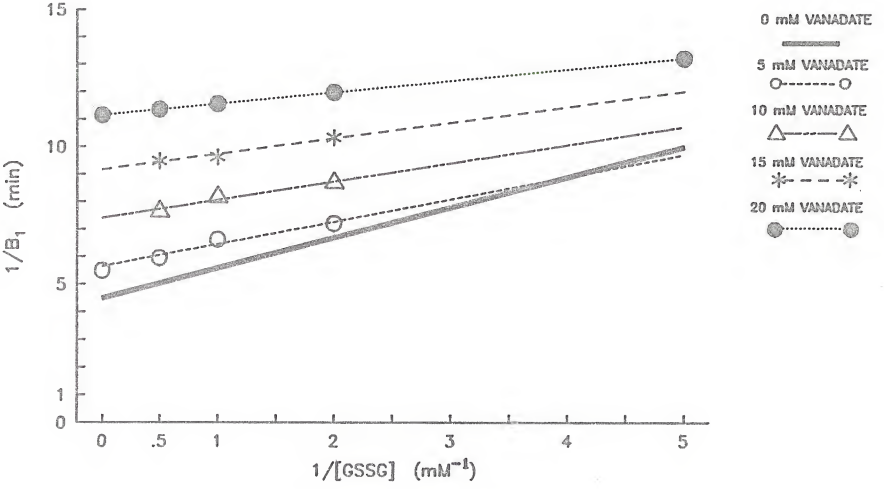


Figure 32. Double-reciprocal analysis of GSSG-R activity with increasing levels of vanadate. Levels of metal and GSSG in the mixture are indicated in the figure. Assay conditions are described in Figure 30.

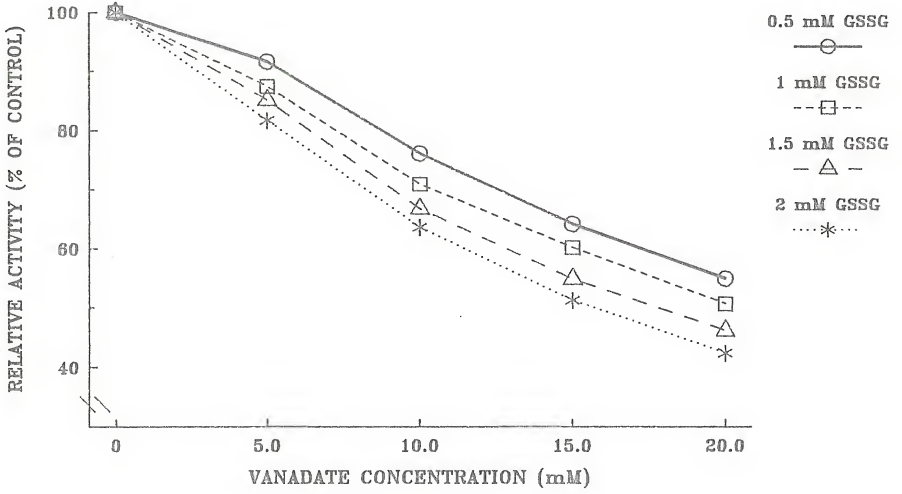


Figure 33. Relative activity of GSSG-R as a function of vanadate and GSSG levels. All conditions are as described in Figure 30.

the metal, when the vanadate was introduced there was a rise in the enzyme activity. When vanadate levels of 3 mM or greater were used, the activity reached a plateau (Figure 34) indicative of some inhibition at the substrate level.

The complexation of vanadate with NADP(H) was outlined in the glucose-6-phosphate dehydrogenase study. When GSSG was mixed with increasing levels of vanadate, an immediate yellow color appeared and persisted. At higher levels of metal, from 50-100 mM, an orange color formed immediately. The absorbance spectra of these metal:GSSG mixtures display a new absorbance peak in the 410-440 nm region (Figure 35). With increasing levels of GSSG at a fixed level of vanadate, this new peak underwent a bathochromic shift. This coloration and movement of the peak to higher wavelengths was indicative of charge-transfer complex formation.

The degree of complexation as well as the strength of the binding between vanadate and GSSG was determined using Scatchard analysis. From the changes in the absorbance at 420 nm with increasing levels of vanadate, the ratio of bound vs free metal was determined. The Scatchard plot that resulted was biphasic (Figure 36), and yielded binding constants for two non-identical sites on the new complex of $4.55 \times 10^2\ M^{-1}$ ($pK_a = 2.66$, $n_1 = 4$) and $2.59\ M^{-1}$ ($n_2 = 8$), respectively.

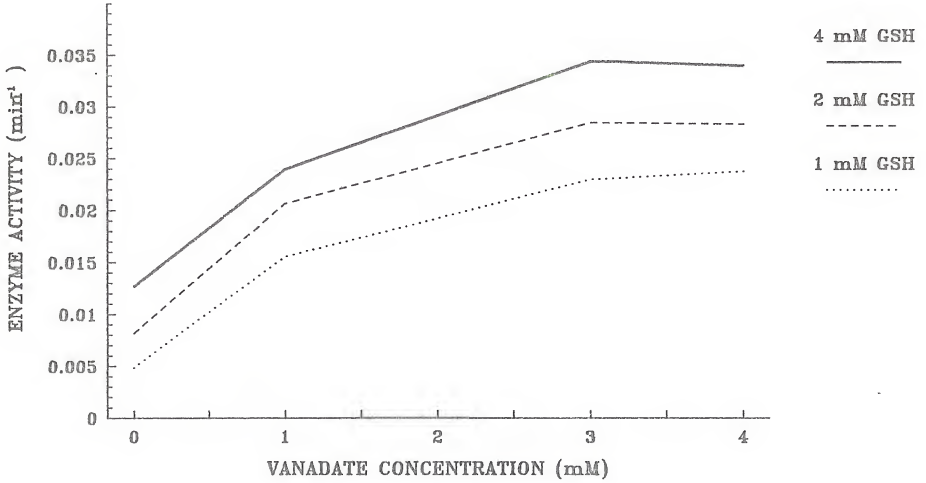


Figure 34. GSSG-R activity as a function of reduced glutathione and vanadate. Activity was measured by the rate of disappearance of NADPH/min at 340 nm following initiation of reaction with 0.17 mM NADPH. Each point was the mean (± SD) of 4 runs at each vanadate level.

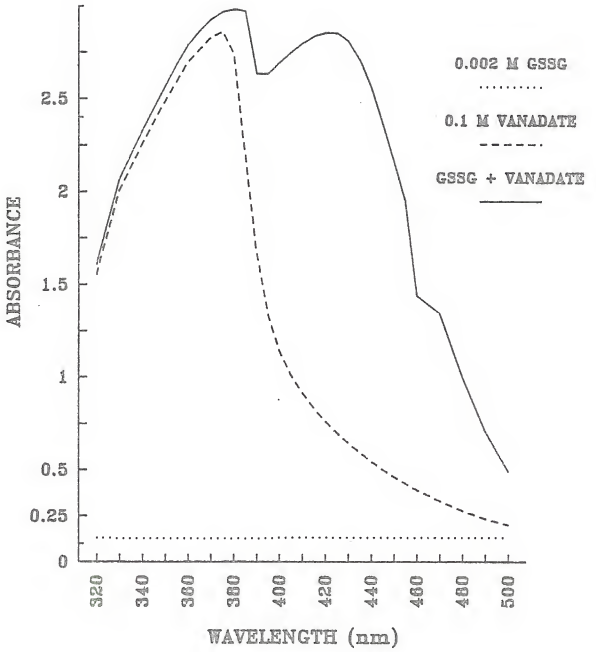


Figure 35. Absorbance spectra of glutathione disulfide in the presence/absence of vanadate ions. Spectra were constructed from measurements of absorbance at 5 nm intervals. Mixture volume (3 ml) contained aqueous vanadate (0.10 M) and 2 mM GSSG. In scans without GSSG, water was used as replacement. Each curve is the composite of 3 scans.

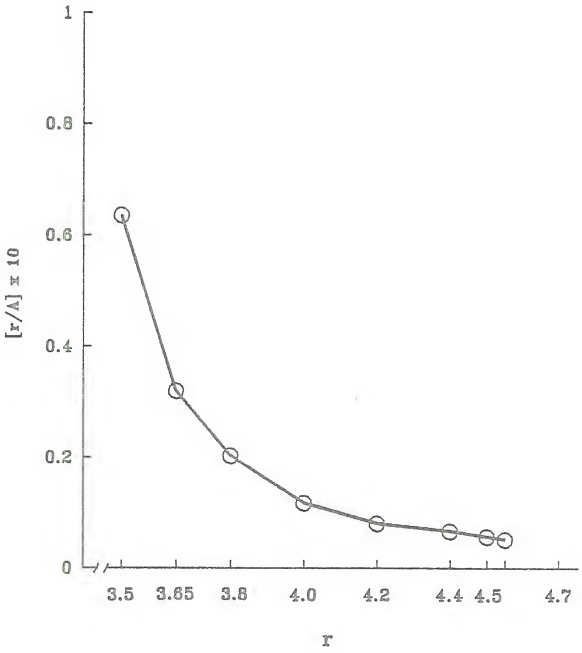


Figure 36. Scatchard analysis of the interaction between vanadate ions and GSSG. The x-axis (r) represents the concentration of bound metal (A_b) in relation to the total concentration of GSSG ligand present, and the y-axis (M^{-1}) is r in relation to the concentration of unbound metal (A_f). Data was generated from the change in absorbance at 425 nm while varying the concentrations of vanadate in the presence of a fixed amount of ligand.

Discussion

In the first portion of this chapter, the effect of ammonium metavanadate on the enzyme glucose-6-phosphate dehydrogenase (G6PDH) was analyzed using a cell-free system. This study also examined the effect of vanadate on glutathione reductase (GSSG-R), which in the cell is dependent on the dehydrogenase. The reductase utilizes the reducing equivalents provided by the G6PDH reaction. Since vanadate inhibited G6PDH through complexation with the $NADP^+$ cofactor, the GSSG-R would be expected to display similar inhibition due to NADPH being one of its substrates.

To better understand the possible mechanisms of inhibition by vanadate, a discussion of the reaction mechanism is required. The enzymatic conversion of oxidized glutathione (GSSG) to two equivalents of reduced product (GSH) proceeds through an ordered sequential mechanism (Staal and Veeger, 1969; Icen, 1971) while consuming one NADPH equivalent. The reductase active site contains a disulfide moiety in proximity of a prosthetic flavin group. This flavoenzyme undergoes complex intramolecular redox reactions in order to bind the substrate and release the product (Figure 37).

The NADPH acts as the electron donor and is bound to the native enzyme (E, State I) resulting in the opening of the disulfide linkage via electron transfer and reduction to thiol and thiolate moieties. Following the two electron transfer, a reduced enzyme is present (F) which contains the

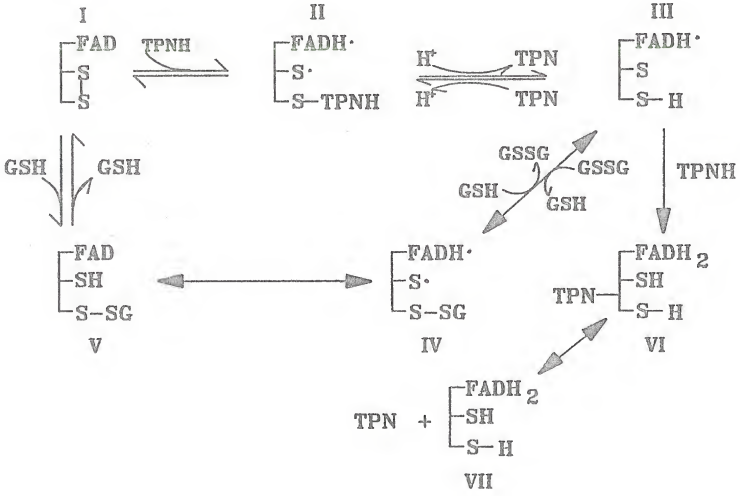


Figure 37. Proposed mechanism of GSSG-R catalytic activity.

bound pyridine ($F:NADP^+$, State II). With $NADP^+$ bound, there is a conformational change at the active site that enhances the binding of the GSSG substrate (Moroff and Brandt, 1975). Staal and Veeger (1969) proposed that the oxidized glutathione binds the reduced enzyme to yield a transient ternary complex ($F:NADP^+:GSSG$). The GSSG undergoes a rapid disulfide interchange reaction with the reduced thiol and this splits off the first reduced glutathione molecule while the other glutathione molecule forms a mixed disulfide with the enzyme (State IV). Through rapid electronic rearrangement, the flavin donates an electron (acquired from NADPH) back to the neighboring sulfur radical to yield a fully reduced thiol site (State V). An intramolecular nucleophilic attack on the glutathione-bound sulfur then splits off the second molecule of GSH and regenerates the native enzyme (E).

During the reaction, the binding of the GSSG induced a second conformational change at the active site and this enhanced the dissociation of the oxidized pyridine product ($NADP^+$). The result of the reaction was one in which two electrons were donated by NADPH and two electrons were subsequently transferred to the GSSG. Similarly, in the presence of an excess of NADPH, a four electron reduction may occur (State VII) and the reaction progresses at a slower rate using the above mechanism. The fully reduced flavin molecule may then act as an electron donor later in the reaction when NADPH levels are not quite as high (Bulger and Brandt, 1971).

Based on the known oxidative role of vanadate (Snyder and Cornatzer, 1958; Macara et al., 1980; Ramasarma et al., 1981), the above reaction mechanism provided several possible sites for vanadium interference. Oxidation of NADPH, the newly formed thiol groups or the flavin group seem to be likely points for study. Flavin is known to be oxidized by pentavalent vanadium (Gallagher and Riechel, 1982) and was not studied here. In the first portion of this chapter, it was shown that vanadate oxidized NADPH to $NADP^+$. Vanadate complexation with NADPH was also observed and this likely affected the formation of the reduced enzyme (F) as well as the E:NADPH complex. Therefore, besides the effects of vanadate on enzyme activity, this study also focussed on the effects of vanadate on the oxidized glutathione substrate.

It was previously established that the maximal activation of the GSSG-R system occurred at pH 7.5-8.0 in the presence of a 0.1 M ionic concentration (Icen, 1967; Moroff and Brandt, 1973). The type of ion present was a determining factor in the degree of activation up to 0.1 M, as well as in the extent of inhibition when anionic concentrations were above this level. Halides were often the most potent inhibitors with 50% inhibitory concentrations (IC_{50}) ranging from 90-180 mM. These values were thought to be dependent on halide atomic radii, with iodide being strongest and fluoride weakest. However, steric factors could not be the primary factor since acetate, a larger molecule than iodide, was a weak inhibitor.

In this study, a similar comparison was performed using metal oxides. Kinetic studies using vanadate, iodate, tungstate, and molybdate gave rise to an inhibitor order of strength of IO_3^- > MoO_4^- > VO_3^- >> WO_4^- (data not shown). The bond lengths of these anions differ only by 0.06 angstroms (van Etten et al., 1974). It was more likely that the very strong inhibition by iodate was due to disproportionation to periodate, IO_4^-, which was a potent oxidizing agent (Purcell and Kotz, 1977). When vanadate was present in the reductase system, there was a concentration-dependent decrease in activity although the anionic concentration was maintained at 0.1 M by the addition of phosphate ions. At 0.1 M vanadate, the reaction was completely inhibited. An IC_{50} of 19 mM was calculated for vanadate based upon the observed activity as a function of vanadate present.

In an attempt to determine the possible mechanism of vanadate inhibition, Lineweaver-Burk analysis with GSSG as the varied substrate could not be adequately performed, as the double-reciprocal plot showed a convergence of the data points in the (+X, +Y) region. Moroff and Brandt (1973) were able to demonstrate that the inhibition with halides was competitive and noncompetitive with respect to the NADPH and GSSG, respectively. The double reciprocal plot in this study using NADPH as the varied substrate indicated mixed-type inhibition. Other data analysis, including Dixon and Hanes-Wolff plots ($[S]/B_1$ vs $[S]$) indicated that the vanadate displayed competitive inhibition regarding the

NADPH, with a calculated K_i of 1.2 mM. The complexation of vanadate with the pyridine cofactor led to competitive inhibition in the G6PDH study, and so it was expected that a similar result would be obtained with the reductase. What was clear from these analyses was that other interactions were occurring with the GSSG since the data obtained using GSSG as a varied substrate did not lend themselves to conventional interpretation.

If the mechanism of vanadate inhibition with respect to GSSG was at the level of the enzyme and was also reversible (i.e competitive), then an increase in the GSSG levels should overcome the inhibition. When GSSG levels were increased in the presence of fixed levels of vanadate there was an increased inhibition rather than reversal. Moroff and Brandt (1975) detailed the inhibition by higher levels of GSSG when the ionic concentration was optimal. They described the effects of excess GSSG on the turnover time for the NADPH as well as on the regeneration time of the active site. But the vanadate data in the present study was analyzed in comparison with vanadium-free reaction systems and so any contribution to inhibition by GSSG was already taken into consideration. This indicated that some inhibition of the reaction was occurring at the substrate level. Therefore, an examination of the complexation of vanadate with both reduced and oxidized glutathione was performed.

Complexation of vanadate with GSH had been previously documented (Delfini et al., 1985; Legrum, 1986). In these

studies, complexation occurred following a reduction of the pentavalent vanadium to the tetravalent vanadyl ion. This species then complexed with an excess of GSH to yield colored compounds which were 2:1 complexes of GSH:vanadyl. In the present experiment, there was an excess of vanadate as compared to GSH, and no colored complexes were observed. Similar studies with cysteine indicated no complexation although other investigators have observed such with an excess of the ligand (Sakurai et al., 1981). In this experiment, colored compounds were only detected when oxidized ligands, cystine or GSSG, were employed.

A yellow coloration immediately formed upon mixing the clear aqueous vanadate and ligand mixtures. When the ligand (GSSG or cystine) concentration was increased, the solution became orange. This suggested of ligand to metal charge transfer complexation and this was confirmed spectrophotometrically. When 0.1 M vanadate was mixed with 2 mM GSSG, a new absorbance peak absent in the indiviual spectra was observed at 410-430 nm. If the level of GSSG was increased further, the peak underwent a bathochromic shift. These two observations were conclusive indications of charge-transfer complexation in the system. Similar results were obtained with mixtures of vanadate and cystine.

The complexes of excess GSH and cysteine with vanadium occurred primarily through the terminal carboxyl groups (Delfini et al., 1985) or through the thiol and amine moieties (Legrum, 1986). Nechay et al. (1986) determined

that the pKa of such complexes was 4.30 at room temperature. In this study, when the GSH levels were adjusted so that the concentrations of these binding sites were equivalent to that in the complexing GSSG, still no coloration was observed. This implied that the complexation was occurring primarily through the disulfide linkage in both GSSG and in cystine. This was possible because the electron-rich region of the molecule contained a total of eight non-binding electrons from both sulfur atoms which were available for interaction with the vanadium empty valence shells.

Vanadate contained the pentavalent vanadium atom, so that the electronic configuration in the resting state was $3d^{o}\ 4s^{o}$. This meant that the vanadium had a low energy ground state that was designated $^{1}S_{0}$ non-degenerate. This then allowed for energetically favorable transitions of electrons from the sulfur atoms (also $^{1}S_{0}$ at resting state). Also contributing to this favorable situation, was the high field splitting energy of the pentavalent vanadium. This would mean that any electron entering the outer shells would be forced into a low energy level (designated t_{2g}^{*}) for binding. This forced localization would assist in a rapid filling of the outer orbitals with the least expenditure of energy. As a result of the high splitting energy, low spin octahedral complexes would result; it has already been shown that aqueous vanadate preferentially forms octahedral complexes (Chasteen, 1983). The thermodynamic and steric factors were most favorable to the complexation occurring

through the disulfide region. From the GSH studies, it appeared that the other possible ligand sites had a minor role in the complexation of vanadate with GSSG.

From an analysis of the absorbance of the charge-transfer peak with metal ion levels being varied, Scatchard analysis was employed to determine the strength of this complexation. The resulting Scatchard plot was not linear, indicating that more than one class of binding sites existed on the GSSG molecule. The biphasic curve yielded a binding constant of 4.55×10^2 M^{-1} for the first class of sites with an n value of 4. This implied that 4 vanadate ions were bound to each molecule of GSSG at these sites. After accounting for the contribution to the total binding from the first class of sites, the second class was determined to have a binding constant of only 2.59 M^{-1}. Each of these low K_a values was consistant with a charge-transfer complex.

The effect of vanadate complexation with GSSG as a contributing factor toward enzyme inhibition was demonstrated again by replacing GSSG with GSH in the reaction. The oxidative action of the vanadate was demonstrated in that a non-utilizable substrate (GSH) was converted to the active substrate (GSSG) and enzyme activity was observed. However, at higher levels of metal, the activity became relatively constant and low, regardless of the initial level of GSH added. The newly formed GSSG was most likely bound up with metal ions and enzyme activity was again blocked.

Although not addressed in this study, the possibility of vanadate causing inhibition by direct interaction with the enzyme active site has its basis in studies with other inorganic ions. Copper (Rafter, 1982), gold (Shaw, 1979 and 1980) and arsenic (Williams, 1976) have been shown to bind to active site thiols with subsequent loss of enzyme activity. Vanadate would likely bind to active site thiols, and if sterically possible, might even complex with the disulfide moiety prior to NADPH activation.

As observed with the yeast G6PDH, the effects of vanadate on the substrates and products of the GSSG-R reaction played a major role in the reaction kinetics observed. Because these enzyme inhibition experiments were run under optimal conditions in a cell-free environment, it might be that the observations here would not apply to the macrophage system discussed in Chapter IV. Differences in interpretation of data have resulted from studies using cells from nickel-exposed animals (Camner et al., 1978; Jarstrand et al., 1978) and cells exposed to nickel after harvesting from untreated hosts (Graham et al., 1975).

The purpose of this series of experiments was to provide possible mechanisms for the enzyme inhibition that was observed in the mouse macrophages from vanadium-treated mice. What was obtained was not only an explanation for enzyme inhibition by vanadate, but an enhancement of the understanding of the interactions of this agent with several biologically important molecules.

CHAPTER VI
SUMMARY

Previous studies showed that subchronic exposure of mice to ammonium metavanadate resulted in immunomodulation. Decreased macrophage phagocytosis of inert microspheres and a sharp reduction in resistance to challenge with the bacterial pathogen Listeria monocytogenes suggested that the vanadium immunotoxicity was mediated through a disruption of cell-mediated immune functions. In this dissertation, results from the studies described in Chapter III indicated that vanadium-pretreated mice were unable to clear a sublethal dose of Listeria from the peritoneal cavity. Clearance mechanisms in the liver and spleen were also disrupted by vanadate exposure. Increased bacterial burdens in each of the three sites suggested that vanadate disrupted resident macrophage phagocytic functions and/or intracellular killing and possibly accessory cell recruitment to help eliminate the organism. An earlier study had shown that vanadate treatment resulted in a time- and dose-dependent atrophy of the thymus. Such an effect by vanadate would indicate depletion of T-cells rather than an effect on antigen processing and stimulation of naive T-cells by macrophages.

The present study demonstrated that resident peritoneal macrophages from vanadium-treated mice ingested less opsonized Listeria than cells from control mice. Even after ingestion had occurred, these cells were less capable of killing the bacteria. Because Listeria parasitized the macrophages, reduced intraphagolysosomal killing allowed replication of macrophage-resistant progeny. The progeny eventually killed the host cell and reinfected other macrophages as well as other sites in the animal host. This explains the dramatic rise in recovered Listeria at the site of infection as the length of the infectious period increased. A direct effect of vanadium on Listeria growth and replication was not indicated, as the population profiles in infected cells did not correlate with host-pretreatment.

To elucidate the possible mechanism by which vanadate affected macrophage phagocytic and intracellular killing processes, studies of the activities of glucose-6-phosphate dehydrogenase, glutathione reductase, and glutathione peroxidase were undertaken. These enzymes, in conjunction with the glycolytic pathway, contribute to the production of energy required for phagocytosis. In the peritoneal macrophage, they are also utilized for activated oxygen metabolite formation and final detoxification. As outlined in Chapter IV, the freshly harvested peritoneal macrophages from vanadate-treated mice displayed dose-dependent decreases in the activities of each enzyme.

Glucose-6-phosphate dehydrogenase is the rate-limiting enzyme of the hexose monophosphate shunt. Along with glycolysis, this shunt is the means by which murine peritoneal macrophages form most of the reducing equivalents used for phagocytic energy. Decreased enzyme activity at this level will result in less energy for active phagocytosis. The reductase has been shown to be essential for the amplification of shunt activity after phagocytosis is initiated (Strauss et al., 1969), as well as to aid in the rapid production of activated oxygen metabolites. The decrease in reductase activity will not only limit the amplification process but also will affect the ability of the cells to produce superoxide anion. Because the mouse peritoneal macrophages lack cationic proteins and myeloperoxidase for halogen activation (both employed in bacterial killing), superoxide anion and its dismuted product, hydrogen peroxide, are critical for the killing of Listeria. Thus, the lowered reductase activity after vanadate treatment resulted in decreased formation of superoxide anion and consequently contributed to the survival and ultimate intracellular replication of Listeria.

Mouse peritoneal macrophages are also deficient in catalase, and thus the role of glutathione peroxidase in cellular peroxide removal is enhanced. Of the three enzymes studied, glutathione peroxidase appeared most resistant to the effects of vanadate treatment. Normally, reductase activity is needed for the continuing function of peroxidase

in the glutathione redox cycle. With reductase activity reduced by vanadate treatment, there should have been decreased levels of reduced glutathione as the peroxidase continued to function. Increased levels of oxidized glutathione and decreases in free reduced glutathione were observed, but the total glutathione pool was not affected by vanadate exposure. An imbalance in the ratio of these glutathione forms had previously been linked to macrophage dysfunction (Holmes-Gray et al., 1971) and likely contributed to the observed decreased handling of Listeria.

Disruptions in macrophage morphology and the ability to attach to surfaces were also examined to determine their contributions to the decreased phagocytic uptake of Listeria. Results showed that vanadate-pretreatment did not cause any changes. Previous in vitro studies with vanadate detailed changes in macrophage morphology following metal exposure (Waters and Gardner, 1975). It is likely that the levels of vanadate encountered by the macrophages in this in vivo study were below that which caused the reported changes in cell architecture. A study of vanadium accumulation in the peritoneal macrophages is currently in progress, since the immunotoxicity of many metals has been related to their uptake by macrophages with subsequent disruption of biochemical function and immune activity.

Since the effects of vanadium on macrophages appeared to focus on phagocytic function and intracellular killing of bacteria, in vitro enzyme studies related to these functions

were performed. The studies described in Chapter V with purified glucose-6-phosphate dehydrogenase and glutathione reductase indicated that inhibition of each was due to metal interaction with the substrates/cofactors as opposed to direct interaction with the enzyme.

Inhibition of the dehydrogenase was the result of vanadate complexation with the adenine ring system of the $NADP^+$ cofactor so that the active enzyme tetramer could not form. Inhibition of glutathione reductase was likely due to charge-transfer complexation of the metal oxide with the oxidized glutathione substrate. Previous reports of inhibition of these enzymes with other inorganic compounds have suggested that inhibition was the result of complexation with enzyme active site thiols or disulfides. Although not studied here, the chemical nature of vanadate would not preclude a similar effect in addition to the interaction with substrates. As noted in the text, many studies have demonstrated the ability of vanadate to interact with glutathione and nicotinamide substrates in cell-free systems. Only a few studies have reported actual binding of vanadate to intracellular ligands (Macara et al., 1980; Nechay et al., 1986) and these have mainly described interactions in terms of oxidation/reduction reactions.

Thus, the interaction of vanadate ions with the substrates needed for oxygen activation and, in part, for the production of energy for phagocytosis in mouse peritoneal macrophages resulted in subsequent inhibition of

enzyme activity. Depressed enzymatic activities may contribute to the decreased ability of these cells to phagocytize Listeria or even to kill those organisms that were ingested. The resulting free (non-ingested) Listeria or the more virulent macrophage-resistant progeny may then rapidly reinfect the host leading to death.

Results from this dissertation research showed that vanadium is deleterious to macrophage function and consequently to overall immunity due to the essential role of the macrophage in orchestrating both cell-mediated and humoral immune responses. Many other aspects of cell-mediated immunity need to be addressed. The role of the T-cells and their clonal expansion following macrophage signalling, the production of leukotactic agents such as glutathione-bearing leukotrienes, and the formation/release of progenitor cells from bone marrow following host exposure to vanadate need further study.

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BIOGRAPHICAL SKETCH

Mitchell David Cohen was born on September 26, 1959, in Nyack, New York. He graduated from Spring Valley Senior High School as a New York State Regents Scholar. He attended the State University of New York at Albany under a Frank L. Gannett Scholarship, and received his Bachelor of Science degrees, cum laude, in Spanish literature and in chemistry in 1981. He entered the Department of Food Science and Human Nutrition at the University of Florida in August, 1981, and received his Master of Science degree in 1984. Mitchell expects to receive his Doctor of Philosophy degree from the Food Science and Human Nutrition Department in August of 1988. During the period of his doctoral research, he was awarded a USDA Fellowship for three years, and received honors from the societies Alpha Zeta and Sigma Xi for outstanding graduate research in the agricultural sciences and within the university, respectively. Upon completion of his degree, Mitchell will take up a post-doctoral position at the New York University Medical Center, Institute of Environmental Medicine, and then a career in teaching/research in academia.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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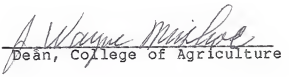
August, 1988



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